REMARKS

Any fees that may be due in connection with filing this paper or with this application during its entire pendency may be charged to Deposit Account No. 02-1818. If a Petition for extension of time is required, this paper is to be considered such Petition, and any fee charged to Deposit Account No. 02-1818.

The Response and Amendment, mailed June 23, 2009, is incorporated herein in its entirety.

In the Response, Applicant referred to numerous references in support of arguments made therein. With the Response, Applicant provided copies of many of the references (24 documents) for the Examiner's consideration. Some references were not provided. Accordingly, provided herewith are additional references (11 documents) also mentioned in the Response and Amendment mailed June 23, 2009.

* * *

In view of the amendments and remarks herein, examination on the merits respectfully is requested.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Serial No.	: 09/724,726	Examiner	: Brent T. Page
Filed	: November 28, 2000	Cust. No	: 77202
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Title : ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

ATTACHMENTS

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Cytogenetic investigations in wheat, rye and triticale. I. Evaluation of improved Giemsa C- and fluorochrome banding techniques in rye chromosomes

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Giemsa's solution and the fluorescent agents Hoechst 33258, DAPI and quinacrine were applied to chromosomes of rye in order to obtain reliable banding techniques. Methodical improvements led to distinct Giemsa C-banding patterns revealing a high number of interstitial bands which allow the discrimination even between closely related rye forms. Hoechst 33258 performed a clear fluorescent banding pattern in rye chromosomes while DAPI showed a similar but less bright fluorescence. Quinacrine proved to be an inadequate tool for rye cytogenetics, although in interphase nuclei regions of brighter fluorescence appeared. Comparing Giemsa C- and Hoechst 33258 banding, the latter could serve for a rapid identification of rye chromosomes, however, Giemsa C-banding is the method at choice for detailed analysis.

INTRODUCTION

The development of banding techniques that allow a differential staining of heterochromatic chromosomal segments made it possible to identify individual chromosomes (Caspersson *et al.*, 1968; Pardue and Gall, 1970). Today such methods are indispensable in modern cytogenetics, not only for karyotype analysis, but also in order to detect structural and numerical aberrations of chromosomes. Moreover, banding techniques are helpful for investigations with regard to evolutionary relationships and in order to reveal differences in chromosomal structure between individuals of the same species or population.

Rye plays a prominent role in plant cytogenetics (*e.g.*, Sarma and Natarajan, 1973; Verma and Rees, 1974; Weimarck, 1975; Lelley *et al.*, 1978; Schlegel *et al.* 1986). Also the increasing impact of hybrid rye and of the interspecific wheat-rye bastard triticale requires accurate knowledge about banding patterns of rye materials.

Banding techniques can be divided into two groups according to the stain which is applicated. Giemsa's solution is the most important of a group of nonfluorescent stains. Banding with a second group of stains, the fluorochromes, is commonly employed to a lesser extent. Fluorescent banding methods for rye chromosomes were first published by Sarma and Natarajan (1973) and Vosa (1974) for the bibenzimidazole derivative "Hoechst 33258" and for quinacrine, however, only the Hoechst technique was successful in differentiating heterochromatic segments. Another interesting fluorochrome is DAPI (4'-6-diamidino-2-phenylindol), which can be successfully applied to stain rye chromosomes (Schlegel *et al.*, 1986).

With respect to the inherent difficulties in reproducing consistent results using existing methods it was our intention to evolve a reliable Giemsa C-banding technique for rye chromosomes which also allows the identification of a large number of C-bands. The second purpose of this work was to develop also a practicable and reproducible fluorescent banding technique by testing the fluorochromes quinacrine, Hoechst 33258 and DAPI.

MATERIALS AND METHODS

Plant materials

Rye cultivar "Halo" (Petkus) was taken as a standard for testing the different banding techniques. In addition we used the rye inbred lines L111, L188 and L281 from the Hohenheim selections. All materials were kindly supplied by Prof. Dr H. H. Geiger, Lehrstuhl für Populationsgenetik, Universität Hohenheim.

Pretreatment

Seeds were germinated in petri dishes at 20°C in the dark. Roots of 1–2 cm were collected and treated with 0·02 per cent colchicine on filter paper for about 2 h. After fixation in ethanol/glacial acetic acid (3 : 1) at 4°C overnight the material was stored in 90 per cent ethanol for up to four weeks.

Preparation of the slides

The ethanol was washed out by distilled water for 10 min. After a mild hydrolysis in 0·1 N HCl at 56°C for 4 min the roots were treated with a solution of 5 per cent pectinase (Rohament P5 from *Aspergillus niger*, 0·45 units/mg, SERVA, Heidelberg) for 1–2 h. Maceration was sufficient if the tips of the roots bent easily but did not fall off. Successively the roots were rinsed in distilled water for 2 × 7 min and incubated in 45 per cent acetic acid. The squashing of the meristematic parts of the root tips (1–2 mm) is described in detail here because this is indeed the most important step of the procedure: In a very small drop of 45 per cent acetic acid on a slide the tissue is plucked to pieces by a preparation needle and a sharp scalpel. One more drop of liquid is added and the slide heated very gently over an ethanol flame. A cover-glass is fitted over the tightened drop and tapped lightly with a needle until the cells have spread, becoming almost invisible. The excess acetic acid is removed from the edges of the coverglass using blotting-paper. A piece of blotting-paper is layed over the slide and carefully squashed once with the thumb. After freezing on dry ice and removing the coverslips the slides were transferred into absolute ethanol for 3–5 min and dried by air.

Giemsa C-banding

Slides which had been air-dried for about one week were put into a filtrated barium hydroxide solution (4 per cent), which was freshly prepared and kept in an oven at 50–55°C. This treatment lasted exactly 10 min. The slides were rinsed thoroughly in running deionized water for 5 min and incubated in a jar of distilled water for 50 min. After 2 × SSC-treatment (0·33 M, 1 h, 60–65°C, *p*H 7) the slides were again rinsed in distilled water 2 × 5 min and put into a staining jar with 3 per cent Giemsa's solution (MERCK, Darmstadt) diluted with Sörensen's buffer *p*H 6·7. Staining occurs very slowly in about 1–2 days at 20°C necessitating periodic monitoring. If staining was satisfactory the slides were rinsed carefully for 10–20 s in distilled water and mounted in Euparal (CHROMA, Stuttgart) after drying by air for one day.

Hoechst 33258 banding

For Hoechst banding the air drying of the squash preparations should not exceed 2–3 days. The slides were rehydrated by ethanol steps (70, 50, 30, 0 per cent). Five minutes was enough for each step. After a pre-incubation in McIlvaine's buffer of *p*H 5·5 for 10 min staining took place 10–30 min in 2 μg/ml Hoechst 33258 dissolved in the same buffer. To obtain the desired concentration it was useful to dilute aliquots of a stock solution of the fluorochrome (100 μg/10 ml distilled water). The exact staining time had to be evaluated using control preparations for the respective staining series. To remove surplus stain the slides were rinsed in McIlvaine's buffer *p*H 5·5 for 2 × 1 min and distilled water 2 × 2 min, briefly dried by blowing air over the surface of the slides, immediately mounted with 60 per cent sucrose dissolved in distilled water, and kept in a cool place overnight. The coverslips were edged by colourless nail varnish. Preserved in this manner the preparations can be stored in the dark at 4°C for more than 6 months until examination under the fluorescence microscope.

DAPI banding

The method for DAPI was the same as described for Hoechst 33258 with the following exceptions: The ethanol steps were replaced by a 15 min incubation in distilled water and the concentration of this fluorochrome was 1 μg/ml. Staining time was 10–15 min and the durability of the preparations several weeks.

Quinacrine banding

Air drying after squash preparation had to be strictly avoided. After the slides had been frozen on dry ice to remove the coverslips they were placed into a dish of glacial acetic acid for 20 s and then transferred into absolute ethanol for 5 min. Staining took place in a solution of 0·25 per cent quinacrine hydrochloride in absolute ethanol during 10–20 min. The slides were washed 3 × 2 min in 96 per cent ethanol, 2 × 3 min in distilled water, and for short-term examination without air-drying mounted in distilled water. For long-term observation they could be preserved by mounting in 60 per cent sucrose after quickly blowing away the water from the surface of the slides by com-

pressed air. By no means should the preparations be allowed to dry up. The preparations were durable for at most 1–2 days.

Fluorescence equipment

The optical outfit consisted of a ZEISS (Oberkochen) microscope WL provided with an epifluorescence equipment and a mercury light source HBO 50 W. The filters are specified in table 1.

Table 1 Filter combinations for fluorescence studies

Filters	For Hoechst/DAPI	For quinacrine
Excitation filter	UG 1/3	BP 433
Beam splitter	FT 420	FT 460
Barrier filter	LP 418	LP 470

Photography

Microphotographs of fluorescent banding were taken on an ILFORD PAN F film, for Giemsa C-banding on AGFAORTHO 25 film, using a ZEISS C35 camera.

RESULTS AND DISCUSSION

The Giemsa C-banding patterns resulting from the technique described lead to an increased number of interstitial bands (fig. 1). Connections between these and the chromomere pattern of pachytene chromosomes can be implied according to Nagl (1976). Further investigations about early mitotic metaphase and meiotic pachytene stages by means of Giemsa C-banding will give more evidence about this question. In comparison with other exemplary publications since Vosa (1974) a progress in the detected amount of C-bands in the genome can be noticed (table 2). It is our opinion that this development has still not reached its end, so that for all we know the detection of more than 80 C-bands in the haploid rye genome seems to be possible. However, an achievement of a uniform Giemsa C-banding method, hitherto different in any publication, should be taken into account.

Table 2 Maximum number of interstitial and telomeric Giemsa C-bands in the haploid genome of rye given in a sample of publications (modified after Hesemann *et al.*, 1986)

Authors	C-bands
Vosa (1974)	23
Hadlaczky and Koczka (1974)	25
Giraldez *et al.* (1979)	27
Schlegel *et al.* (1986)	28
Badaeva *et al.* (1986)	29
Semenov and Semenova (1982)	30
De Vries and Sybenga (1976)	34
Lelley *et al.* (1978)	34
Hesemann *et al.* (1987)	59

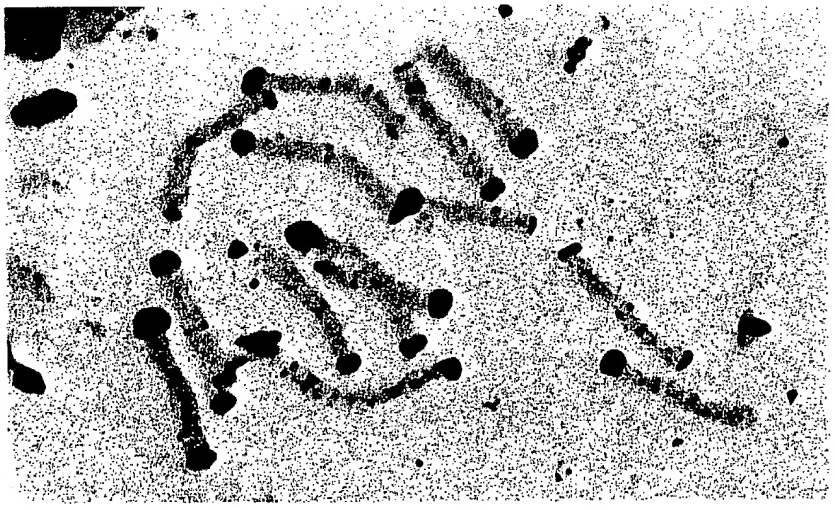

Figure 1 Giemsa C-banding in a metaphase cell of L281.

With the co-operation of all rye cytologists, who are interested, such a standard technique may be established on the basis of the presented method. In this context we also welcome the standard band nomenclature for rye chromosomes proposed by Schlegel *et al.* (1986).

During our experiments to establish a reproducible C-banding method we observed the following steps of the procedure to be of great relevance to success or failure:

—During HCl-hydrolysis, it is essential to strictly observe the appointed temperature given for an alcohol thermometer. Too low temperature results in an insufficient clearing of the cytoplasm, too high temperature results in complete failure of the banding.

—Enzymatic maceration must be adapted to the specific charges of pectinase delivered by the producer. The enzyme should be washed out carefully, if not, banding will be inhibited.

—Squash preparation causes the most deviations of all. Leaving all other steps constant and only changing the persons doing the squash preparation often leads to more different results than varying any other step.

—Air drying of the slides after squash preparation is necessary for at least five days. A longer period of maturation will shorten the staining time.

—Denaturation with barium hydroxide is also a delicate step and should be carried out according to the instructions given.

Differentiation even between closely related forms is possible with this Giemsa C-banding technique. For example between cv. "Halo" and the inbred line L188 obvious differences in the banding patterns can be observed, although both belong to the same source population. The chromosomes are presented in fig. 2. Due to homozygosity inbred lines show a more constant banding pattern, although a lower amount of interstitial bands in total, but the C-bands of the heterozygous cultivar "Halo" often seem more inconsistent. These observations correspond well with our results about the polymorphism of rye chromosomes (Hesemann *et al.*, 1987).

Application of the Hoechst 33258 banding technique resulted in specific fluorescent patterns at heterochromatic regions of rye chromosomes (fig. 3). According to our experience fluorochrome banding with Hoechst 33258 is not as susceptible

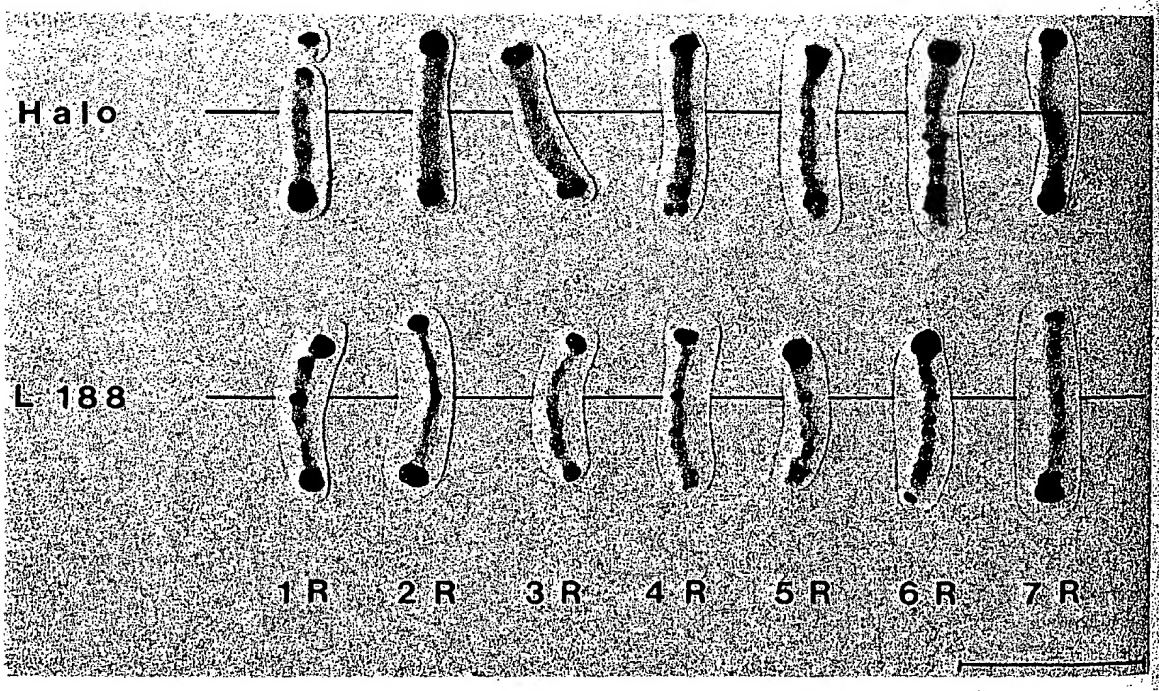

Figure 2 Giemsa C-banded chromosomes of cv. "Halo" and inbred line L188. Bar = 10 μm.

Figure 3 Hoechst 33258 banding in a metaphase cell of cv. "Halo".

methodical deviations as Giemsa C-banding. ne decisive factors of the technique described are follows:

—HCl-hydrolysis should not reach 60°C in temperature, otherwise this would give rise to unsatisfactory banding results. With temperatures of about 60°C the nucleoli will be seen as "dark holes" in the interphase nuclei. Similar observations have been described by Laloue *et al.* (1980) for nuclei of *Nicotiana* and *Asparagus.*

—Rehydratisation of the slides proved to be necessary. For rye chromosomes Sarma and Natarajan (1973) put the air-dried slides directly into the staining solution. This was not successful in our own experiments. Improved results were achieved by using a rehydratisation with ethanol steps and a pre-incubation in distilled water and McIlvaine's buffer (Caspersson *et al.*, 1970; Schweizer and Nagl, 1976).

—Banding techniques with Hoechst 33258 differ widely in staining time, concentration of the stain, solution medium and its *p*H. In our test series for *p*H 4·0 up to *p*H 7·0 the brightest fluorescence was revealed at *p*H 5·5. At *p*H 7 the fluorochrome is unfortunately bound to the cell walls changing into a faint yellow tone at *p*H 4.

—Washing out the surplus stain is necessary to inhibit background fluorescence. On the other hand the rinsing should not last longer than 6 min, otherwise the slides will de-stain and the fading effect will be strengthened.

—The most critical point of fluorescent banding is the choosing of the appropriate mounting medium. Vosa (1974) used distilled water and glycerine mixtures, Das *et al.* (1979) mounted the slides in Sörensen's buffer or in a glycerol buffer mixture. These mediums are sufficient for short-term examination of a few slides. For the investigation of more slides a way of mounting is required which enables preparations of a longer durability. We found moisture chambers not useful for this purpose. The best mounting medium proved to be 60 per cent sucrose. In opposition to Lee and Collins (1977), who dissolved the sugar in McIlvaine's buffer for their Q-banding experiments, we detected that for Hoechst 33258 a solution in distilled water brings about the best vision under the fluorescence microscope.

—The slides should be irradiated only after a period of cool storage in the dark for at least one night or longer because immediate examination gives rise to an intensified fading effect.

The Hoechst 33258 banding technique resulted in two types of fluorescent bands. Telomeric bands revealed a strong and bright fluorescence and interstitial bands showed a faint but distinct fluorescence for a few characteristic bands of the rye

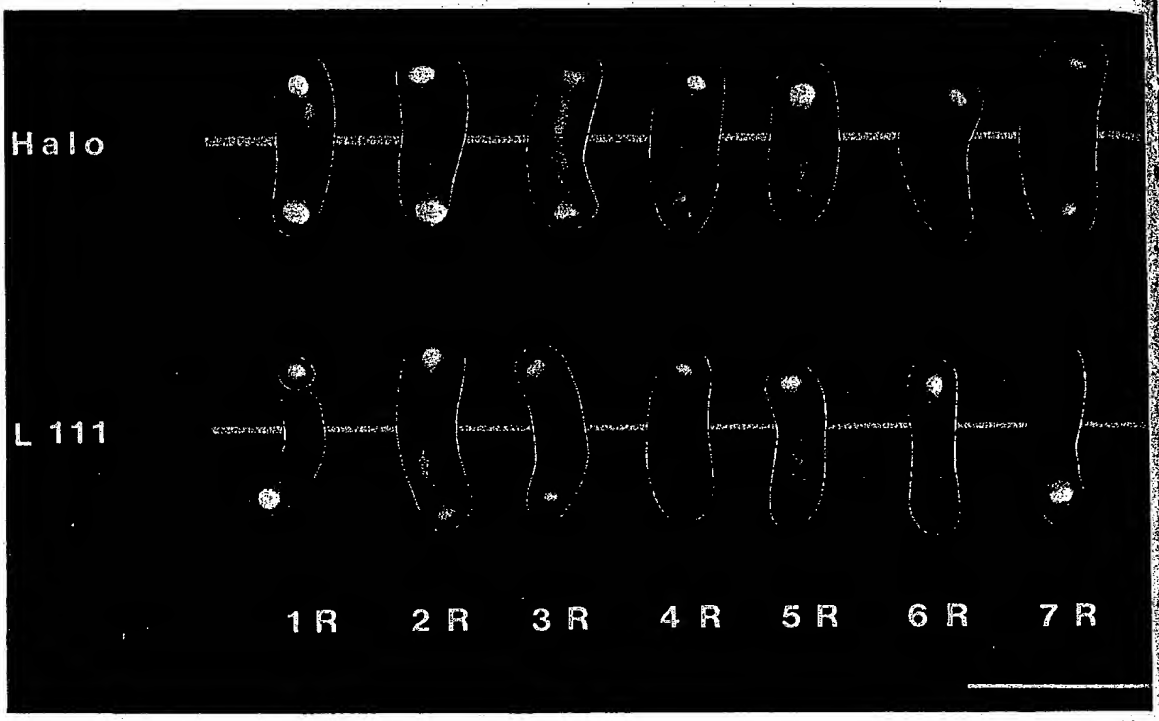

Figure 4 Hoechst 33258 banded chromosomes of cv. "Halo" and inbred line L111. Bar = 10 μm.

genome. In the chromosomes of Halo and L111 after Hoechst staining (fig. 4) only rough differences are visible particularly with regard to the extent of telomeric bands. Discriminating different rye forms by this type of banding will be difficult, if no fundamental differences in the distribution or amount of telomeric heterochromatin are present. As for Halo and L111, although closely related, the chromosomes 1R, 4R, 5R and 7R could be discerned not only by telomeric but to some extent also by interstitial bands.

For Hoechst 33258 banding Sarma and Natarajan (1973) gave a nomenclature for five different types of chromosomes which can be allied to the standard nomenclature as shown in table 3. The same authors complained that interstitial bands had been found only in early metaphase stages. This cannot be confirmed from our results.

DAPI-banding of rye chromosomes is reported by Schlegel *et al.* (1986) from unpublished results. In our own experiments DAPI showed similar banding patterns as Hoechst 33258 but photodocumentation was embarassed by less bright fluorescence.

Table 3 Five types of Hoechst 33258 banded rye chromosomes as described by Sarma and Natarajan (1973) allied to the standard nomenclature (Sybenga, 1983)

Sarma and Natarajan	Standard nomenclature
Type A	2R/3R
Type B	6R
Type C	1R
Type D	7R
Type E	4R/5R

Attempts of a Q-banding method for rye chromosomes were described by Sarma and Natarajan (1973) and Vosa (1974). They failed to establish a Q-banding pattern. Also our own test series were not successful in achieving a clear banding pattern in metaphase chromosomes, however, in interphase kernels (fig. 5(d)) and partially in prophase chromosomes areas of brighter fluorescence appeared. So Q-banding of rye chromosomes does not seem impossible in future if the following critical points are investigated further:

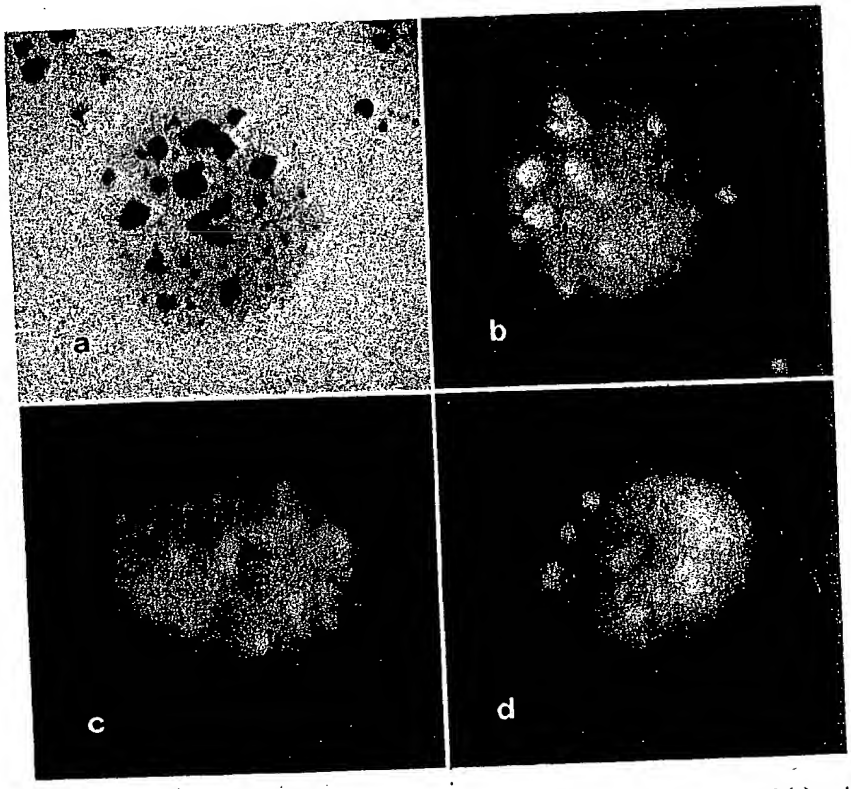

Figure 5 Interphase cell nuclei of cv. "Halo" stained with Giemsa (a), Hoechst 33258 (b), DAPI (c) and quinacrine (d).

—Variation of the HCl-hydrolysis did not affect the results of the quinacrine staining. Kongsuwan and Smyth (1977) replaced this step by a long maceration in 45 per cent acetic acid and attained a Q-banding pattern of *Lilium* chromosomes. They also observed chromosomes surrounded by cytoplasm not being stained differentially since single chromosomes spread outside from the cells revealed a bright Q-banding pattern. However, using the above method we did not observe any banding in rye chromosomes.

—Cellplasm has the negative character to bind the stain, so that background fluorescence will interfere with the possibility of observation. In order to reduce this effect we used a short treatment with glacial acetic acid directly after removing the coverslips.

—Unquestionably the dispense with air drying before and after staining seems to be of advantage.

In comparison of Giemsa and fluorochrome staining of rye cell nuclei the superiority of Giemsa C-banding over all the three fluorochromes is clearly illustrated in fig. 6 for chromosome 1R. As a result of a comparison between Giemsa C- and Hoechst 33258 banding the following evidence can be given:

—Giemsa C-banding leads to a much higher number of bands than Hoechst fluorescent banding, particularly interstitial bands are reduced, which is in part due to the physical and technical limits of fluorescent microscopy.

—Centromeric heterochromatin does not appear as a band with Hoechst 33258. The positions of the centromeres, however, are easily discernable by the constrictions of the chromosomes.

—Without regard to the lower number of bands the Hoechst 33258 banding pattern corresponds well with Giemsa C-bands. Hoechst bands were detected at only the same regions where usually distinct Giemsa C-bands had been observed.

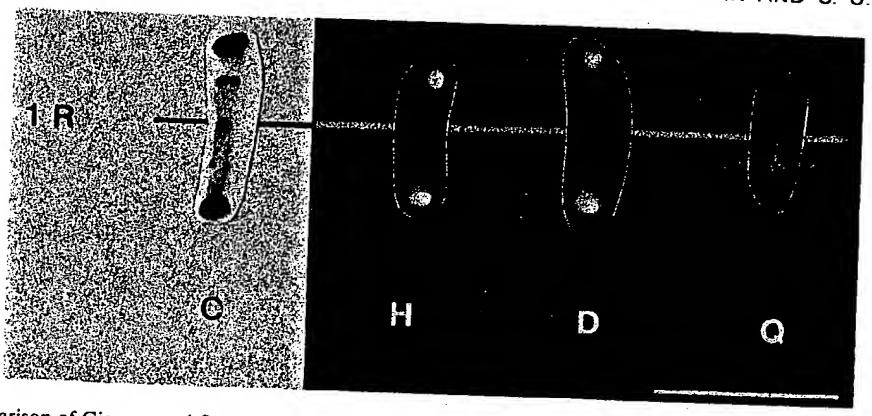

Figure 6 Comparison of Giemsa- and fluorescent staining (Giemsa = C, Hoechst = H, DAPI = D and quinacrine = Q) exemplified by somatic chromosome 1*R*. Bar = 10 μm.

—Two interstitial Giemsa C-bands of the same intensity must not have an analogy in Hoechst banding, so that one or the other band can appear fluorescent possibly due to different types of heterochromatin.
—Telomeric bands in common are comparable with regard to both methods.

In addition to banding patterns of metaphase chromosomes comparison of interphase cell nuclei is of interest to reveal differences between the investigated stains. Figs 5(a) and (b) show that the main chromocentres correspond well between Giemsa C- and Hoechst 33258 banding. DAPI displays a lower bright fluorescence and the chromocentres are not as clearly visible (fig. 5(c)). Quinacrine staining exhibits the lowest number of chromocentres and the nuclei appear more condensed (fig. 5(d)). It would be of interest to know at which sides in metaphase chromosomes these fluorescent regions are located. As mentioned above, it was not yet possible to answer this question.

The described Giemsa technique provides a wide range of interstitial bands, so that just slight differences in chromosomal structure become visible. So far it is the appropriate method for investigating problems of evolutionary or chromosomal importance in detail.

Concerning the fluorochromes, only Hoechst 33258 is recommended yet as a practical tool for rye cytogenetics. It is still not being used far beyond experimental research, however, it could be applicated for example, as a fast method to identify the number of rye chromosomes in wheat-rye substitution or addition lines and in triticale. With regard to this crop, it is also desirable to screen a large number of rye forms in order to evaluate the extent of telomeric heterochromatin. In this manner a contribution could be made to clarify the role of telomeric heterochromatin in triticale cytogenetics.

To sum up, Giemsa C-banding without doubt has the main significance up to now, but at least fluorochrome banding with Hoechst 33258 will be of practical use for special purposes.

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REFERENCES

BADAEVA, E. D., BADAEV, N. S., BOLSHEVA, N. L. AND ZELININ, A. V. 1986. Chromosome alterations in the karyotype of triticale in comparison with the parental forms. I. Heterochromatic regions of R genome chromosomes. *Theor. Appl. Genet.*, *72*, 518–523.

CASPERSSON, T., FARBER, S., FOLEY, G. E., KUDYNOWSKI, J., MODEST, E. J., SIMONSSON, E., WAGH, U. AND ZECH, L. 1968. Chemical differentiation along metaphase chromosomes. *Exp. Cell Res.*, *49*, 219–222.

CASPERSSON, T., ZECH, L., JOHANSSON, C. AND MODEST, E. J. 1970. Identification of human chromosomes by DNA-binding fluorescent agents. *Chromosoma*, *30*, 215–227.

DAS, B. C., RAMAN, R. AND SHARMA, T. 1979. Chromosome condensation and Hoechst fluorescence in meiotic chromosomes of the grasshopper *Spathosternum prasiniferum* (Walker). *Chromosoma*, *70*, 251–258.

GIRALDEZ, R., CERMEÑO, M. C. AND ORELLANA, J. 1979. Comparison of C-banding pattern in the chromosomes of inbred lines and open pollinated varieties of rye. *Z. Pflanzenzüchtg.*, *83*, 40–48.

HADLACZKY, GY. AND KOCZKA, K. 1974. C-banding karyotype of rye from hexaploid triticale. *Cereal Res. Com.*, Vol. 2, No. 4, 193–197.

HESEMANN, C. U., PFEIFFER-SCHAD, B. AND MARTIN, J. 1987. Cytogenetic investigations in wheat, rye and triticale. II.

Extent of heterochromatin polymorphism in cultivars and inbred lines of rye (*Secale cereale* L.), *Plant breeding, 98*, 297-305.

ESEMANN, C. U., PFEIFFER, B., MARTIN, J. AND HAINER, A. 1986. Cytogenetic and cytochemical investigations in primary triticale and its parents. *Proc. Internat. Triticale Symposium, Sydney*, pp. 336-339.

ONGSUWAN, K. AND SMYTH, D. R. 1977. Q-bands in *Lilium* and their relationship to C-banded heterochromatin. *Chromosoma, 60*, 169-178.

ALOUE, M., COURTOIS, D. AND MANIGAULT, P. 1980. Convenient and rapid fluorescent staining of plant cell nuclei with '33258' Hoechst. *Plant Sci. letters, 17*, 175-179.

EE, C. S. AND COLLINS, L. 1977. Q- and C-bands in the metaphase chromosomes of *Drosophila nasutoides*. *Chromosoma, 61*, 57-60.

ELLEY, T., JOSIFEK, K. AND KALTSIKES, P. J. 1978. Polymorphism in the Giemsa C-banding pattern of rye chromosomes. *Can. J. Genet. Cytol., 20*, 307-312.

AGL, W. 1976. *Zellkern und Zellzyklen*. Ulmer Verlag, Stuttgart.

ARDUE, M. L. AND GALL, J. G. 1970. Chromosomal localisation of mouse satellite DNA. *Science, 168*, 1356-1358.

ARMA, N. P. AND NATARAJAN, T. 1973. Identification of heterochromatic regions in the chromosomes of rye. *Hereditas, 74*, 233-238.

SCHLEGEL, R., MELZ, G. AND METTIN, D. 1986. Rye cytology, cytogenetics and genetics—Current status. *Theor. Appl. Genet., 72*, 721-734.

SCHWEIZER, D. AND NAGL, W. 1976. Heterochromatin diversity in *Cymbidium*, and its relationship to differential DNA replication. *Exp. Cell Res., 98*, 411-423.

SEMENOV, V. I. AND SEMENOVA, E. V. 1982. Polymorphism of the rye chromosomes for the heterochromatin bands in some rye varieties and triticales. *Genetika*, Vol. 18, No. 11, 1856-1867.

SYBENGA, J. 1983. Rye chromosome nomenclature and homoeology relationships/Workshop report. *Z. Pflanzenzüchtg., 90*, 297-304.

VERMA, S. C. AND REES, H. 1974. Giemsa staining and the distribution of heterochromatin in rye chromosomes. *Heredity, 32*, 118-122.

DE VRIES, J. N. AND SYBENGA, J. 1976. Identification of rye chromosomes: The Giemsa banding pattern and the translocation tester set. *Theor. Appl. Genet., 48*, 35-43.

VOSA, C. G. 1974. The basic karyotype of rye (*Secale cereale L.*) analysed with Giemsa and fluorescence methods. *Heredity, 33*, 403-408.

WEIMARCK, A. 1975. Heterochromatin polymorphism in the rye karyotype as detected by the Giemsa C-banding technique. *Hereditas, 79*, 293-300.

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Localization of tandemly repeated DNA sequences in *Arabidopsis thaliana*

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Summary

***In-situ* hybridization to interphase nuclei and chromosomes of *Arabidopsis thaliana* (2*n* = 10) shows that there are four sites of rDNA in a diploid nucleus. The sites are located on chromosomes 2 and 4, and the strength of hybridization indicates that copy number is similar at both pairs of sites. Hybridization to trisomic line 4 revealed five hybridization sites. Silver staining of nucleoli demonstrates that all four loci can be active in diploid interphase nuclei. The tandemly repeated probe pAL1 hybridizes near to the centromeres of all five chromosome pairs. In diploid interphase nuclei, 10 sites of hybridization are detected, while 15 are seen in triploid nuclei. The sites of hybridization co-localize with the centromeric heterochromatin visualized by staining DNA with the fluorochrome DAPI. The results demonstrate that molecular cytogenetics can be applied to *A. thaliana* and high resolution physical chromosome maps can be generated. Both probes may be useful for interphase cytogenetics, where they enable chromosome number and aneuploidy to be examined in tissues without divisions. The physical localization of these hybridization sites provides a starting point for linking RFLP and physical chromosome maps.**

Introduction

The physical mapping of DNA sequences on chromosomes is essential for a complete understanding of genome organization. In a few species, including humans, mouse and *Drosophila*, genetic and RFLP (restriction fragment length polymorphism) mapping has been complemented by the cytological study of chromosomes, and *in-situ* hybridization of DNA sequences has been used for physical mapping of the sequence locations (see for example, Disteche *et al.*, 1989). In plants, it is difficult to relate physical chromosome maps to genetical maps, but results from cereals show significant differences with large genetic distances representing small physical distances (Gustafson *et al.*, 1990; Heslop-Harrison, 1991; Snape *et al.*, 1985).

Arabidopsis thaliana has been studied intensively and now has extensive genetic and RFLP maps. The first clear description of the karyotype of *A. thaliana* was made by Steinitz-Sears (1963), and a C-banded karyotype was given by Schweizer *et al.* (1987). However, the small size of the chromosomes (averaging 2 μm long) means that many features of chromosome morphology which are present in other organisms are difficult to visualize. Thus knowledge of the cytogenetics has lagged behind that of molecular genetics.

Despite having one of the smallest genomes of any angiosperm (Bennett and Smith, 1976; Bennett *et al.*, 1982), the genome of *A. thaliana* has a substantial proportion of repetitive DNA. Leutwiler *et al.* (1984) showed that the total DNA (nuclear, mitochondrial and chloroplast) from the plant included 10–14% rapidly annealing sequences, 23–27% middle repetitive sequences, and 50–55% single copy; an unknown but very high proportion of the middle repetitive sequences are from the chloroplast genome.

Two major repetitive nuclear DNA sequence classes have been cloned, which together account for about 10% of the DNA. The largest class is ribosomal DNA (rDNA, Meyerowitz and Pruitt, 1985; Pruitt and Meyerowitz, 1986), which is present in 570 copies per haploid genome. Each rDNA repeat unit is about 10 kb long and the repeat occurs in long tandem arrays, occupying some 8% of the genome. A second repetitive DNA has been cloned by Martinez-Zapater *et al.* (1986). The basic unit is about 180 bp long and there are various small modifications, including some at restriction sites which give repeats of two basic units (including the probe, pAL1, used in the present work). The sequence has been shown to be present in tandem arrays, with a total copy number of about 5000; hence it constitutes 1–1.5% of the genome.

Neither of these repetitive sequences have been located on the major RFLP linkage maps of *A. thaliana* (Chang *et al.*, 1988; Nam *et al.*, 1989) or the genetic map (Koornneef *et al.*, 1983) of the species. In the present work, we aimed to localize the sequences on the chromosomes by *in-situ* hybridization, and examine aspects of their physical organization.

Results

The chromosomes of *A. thaliana* are small and hence cytogenetic analysis is difficult. The method presented

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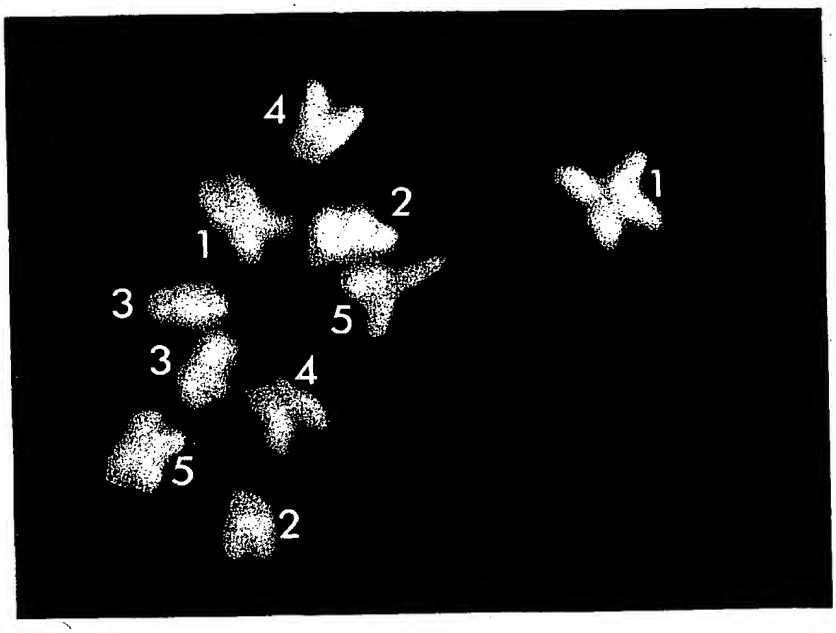


Figure 1. Root tip metaphase chromosomes of *A. thaliana* ($2n = 10$) after DAPI staining. Numbers indicate the linkage group of each chromosome. Magnification × 5250.

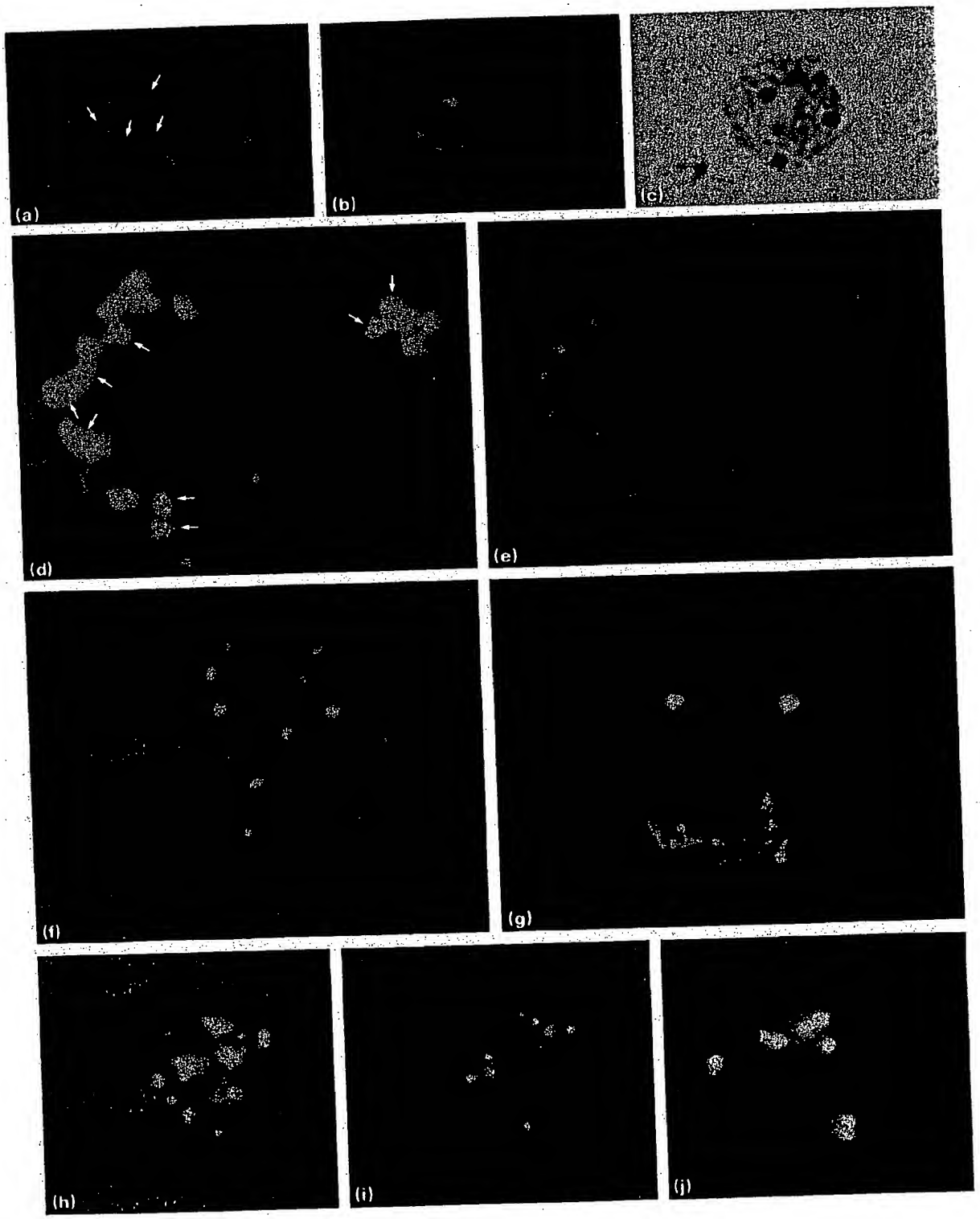
here for chromosome preparation and fluorescence staining with DAPI enabled preparation of good chromosome spreads. Figure 1 shows a metaphase from a root meristematic cell after staining with DAPI. Ten individual chromosomes and the positions of their centromeres could be distinguished. The chromosome designations used here follow the numbers of Koornneef *et al.* (1983) for the genetic linkage map.

In-situ hybridization with digoxigenin-labelled pTa71 (containing the rDNA sequence from wheat, which has high homology to the sequence from *A. thaliana*; cf. Gerlach and Bedbrook, 1979 and Unfried and Gründler, 1990) and the detection of sites of hybridization with fluorescent labels allowed the localization of rRNA genes on metaphase chromosomes and interphase nuclei (Figure 2). Figure 2a shows the 10 metaphase chromosomes after DAPI staining, and Figure 2b the same chromosomes after *in-situ* hybridization with rDNA. Four sites of hybridization, on two pairs of homologous chromosomes can be seen. Analysis of several metaphases showed that rRNA genes were located on the proximal part of the short arm of the acrocentric chromosome number 4 and the distal part of the shortest chromosome number 2. The observation was confirmed by *in-situ* hybridization to tetraploid cells. The 20 metaphase chromosomes after DAPI staining and eight FITC *in-situ* signals show that eight chromosomes include rDNA sequences (Figure 2d and e).

rRNA genes can also be localized at interphase. Four hybridization sites were seen in diploid interphases (Figure 3h), and eight in tetraploid nuclei (Figure 2i). *A. thaliana* trisomic lines were also investigated, and five sites of hybridization were seen in nuclei of trisomic 4 (Figure 2j). Some sites appeared double, perhaps reflecting the replication state of the DNA or partial dispersion of the locus. The nucleus in Figure 2g showed two condensed and two dispersed FITC signals which co-localized with heterochromatin seen in the same nucleus after DAPI staining (Figure 2f). Silver staining of nucleolar-associated proteins showed that up to four silver-positive, dark signals, corresponding to nucleoli (Figure 2c), were visible in single interphases.

Figure 2. Localization of rRNA genes on *A. thaliana* chromosomes by *in-situ* hybridization. Magnification × 2820.
(a, d, f, h) DAPI staining of all DNA following *in-situ* hybridization.
(b, e, g, i, j) The sites of *in-situ* hybridization of an rDNA probe detected with FITC (yellow–green colour).
(c) Silver staining showing four active nucleoli in an interphase nucleus.

(a, b) Diploid metaphase ($2n = 10$). Arrows indicate chromosomes with NORs (a), and *in-situ* hybridization shows the four sites of the rRNA genes (b).
(d, e) Tetraploid metaphase ($4n = 20$). Arrows indicate chromosomes with NORs (d), and eight *in-situ* hybridization signals are visible (e).
(f, g) Diploid interphase nucleus, with two condensed and two dispersed hybridization signals.
(h, i) Tetraploid interphase nucleus with eight *in-situ* hybridization signals.
(j) *In-situ* hybridization signals in trisomic 4 interphase nucleus.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The highly repeated DNA clone pAL1 (Martinez-Zapater *et al.*, 1986) labelled with digoxigenin was also used as a probe for *in-situ* hybridization (Figure 3). It hybridized to the paracentromeric region of all 10 chromosomes (Figure 3b). The strength of hybridization is similar to all centromeric regions and approximately symmetrical on both sides of the centromere (Figure 3d). Figure 3c shows the chromosomes counterstained with DAPI, including the brightly fluorescent heterochromatic block at the centromere.

Interphase nuclei of *A. thaliana* show a number of strong heterochromatic regions (chromocentres), some of which are the centromeric heterochromatin. Figure 3e shows an interphase nucleus with 13 DAPI-positive dots. The same nucleus after *in-situ* hybridization with the probe pAL1 showed 10 homologous sites, corresponding to the number of chromosomes in the diploid cell. In a triploid nucleus, 15 hybridization sites were present (Figure 3k). Hybridization sites were usually discrete and did not cluster in zones within the interphase nuclei.

Figure 3g, h and i show three micrographs of the same nucleus after simultaneous *in-situ* hybridization with two probes, biotinylated rDNA and digoxigenin-labelled pAL1. The signals from the two probes did not co-localize.

Discussion

The karyotype and in-situ *hybridization*

Karyotype analysis of species with small chromosomes is facilitated with fluorescent stains, because the specificity of the stains and the use of a particular wavelength for excitation, and analysis of the one resulting fluorescence wavelength, mean that DNA is clearly visualized. The background is much lower than with conventional stains such as orcein, carmine or Giemsa (Schweizer, 1983). Markers such as centromeric constrictions, heterochromatin, and nucleolar organizing regions (NORs; Schweizer *et al.*, 1987) can be visualized, although the small size of chromosomes (Figure 1) makes detailed morphological studies difficult.

Further markers for chromosome studies can be provided by *in-situ* hybridization of tandemly repeated DNA sequences (Leitch *et al.*, 1991). Detection of hybridization sites with a second pair of wavelengths gives good signal detectability with high specificity. Thus, cytogenetics can be readily carried out with *A. thaliana*.

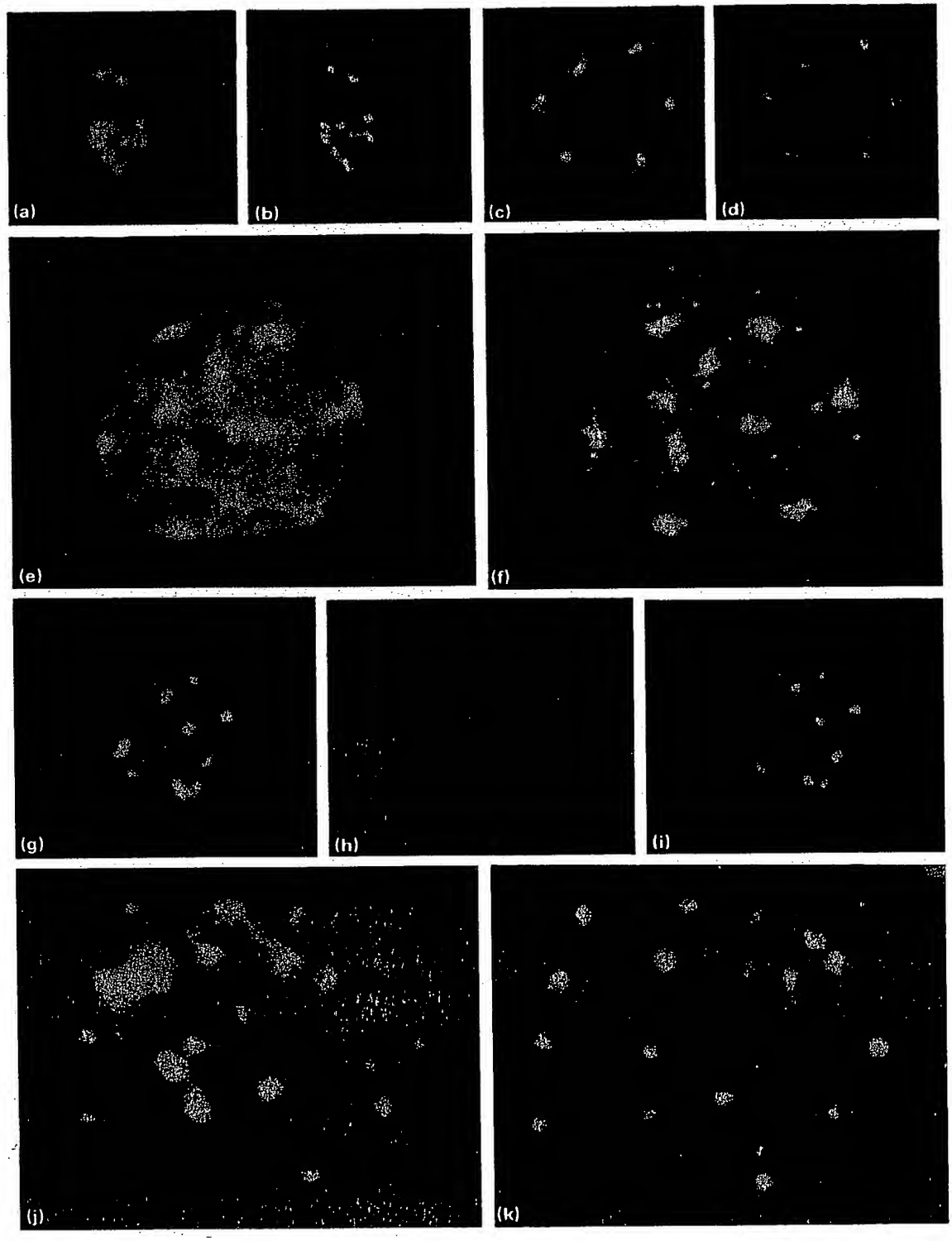
rRNA genes

The results from *in-situ* hybridization show that the rRNA genes are present on two pairs of chromosomes in *A. thaliana*, numbers 2 and 4 (or 4 and 5 using the numbering of Schweizer *et al.*, 1987). Schweizer *et al.* (1987) were able to visualize the rRNA locus on one chromosome pair, number 4 (as seen in Figure 1), from the chromosome morphology in Giemsa-stained chromosome preparations. With the knowledge from the *in-situ* hybridization, a diffuse sub-terminal region can be seen on chromosome 2, as well as 4, which is characteristic of an NOR (Figure 1). Murata *et al.* (1990) reported *in-situ* hybridization with rDNA probes, and also found four sites of hybridization in *A. thaliana*. The signals of *in-situ* hybridization in each interphase nucleus were similar to each other in size (Figure 3h). Although *in-situ* hybridization is not a truly quantitative technique, signal size, at least within a sequence family, does reflect differences in copy number (Maluszynska and Schweizer, 1989; Schwarzacher and Heslop-Harrison, 1991) and hence the two rRNA loci in *A. thaliana* probably have similar numbers of tandemly repeated units in this accession. The number of repeat units at each site is known to vary from genotype to genotype in other plants (Flavell and Smith, 1974).

In-situ hybridization to trisomic lines enables unequivocal assignment of probes to particular chromosomes. Meiotic analysis of trisomic lines has also indicated that there are two NOR chromosome types in *A. thaliana* (Sears and Lee-Chen, 1970). It is perhaps surprising that these major genes have not been located with respect to any RFLP markers, perhaps because the rDNA has few polymorphisms. Investigations using restriction endonucleases with short recognition sequences might detect rDNA polymorphisms, particularly in the intergenic spacers, and hence enable the rDNA loci to be placed on the RFLP maps.

Figure 3. Localization of a highly repeated DNA sequence on *A. thaliana* chromosomes by *in-situ* hybridization. Magnification × 2700.
(a, c, e, g, i) DAPI staining following *in-situ* hybridization.
(b, d, f, i, k) The sites of *in-situ* hybridization of the probe pAL1 detected with FITC (yellow–green colour).
(h) *In-situ* hybridization of rDNA probe and detected with Texas Red (red colour).

(a, b) Diploid metaphase showing *in-situ* hybridization at the centromeric region of all 10 metaphase chromosomes.
(c, d) A partial prometaphase with bright fluorescence at the centromeric heterochromatin (c) and co-localized sites of *in-situ* hybridization (d).
(e, f) A diploid interphase nucleus with brightly fluorescing chromocentres (e), and 10 signals of *in-situ* hybridization (f).
(g, h, i) A diploid interphase nucleus after simultaneous DAPI staining and *in-situ* hybridization with rDNA and pAL1 probes. Four sites of rRNA genes (h) and 10 sites of the centromeric probe pAL1 (i) are visualized, which co-localize with heterochromatin on the DAPI-stained nucleus (g).
(j, k) A triploid interphase nucleus with 15 *in-situ* hybridization sites of the pAL1 probe.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The pattern of rDNA expression is of considerable interest. In many cells, most rRNA genes or even loci are unexpressed, and expression has been correlated with dispersion at the locus during interphase (Schwarzacher and Wachtler, 1983), as shown in Figure 2g where two loci are dispersed. However, many nuclei have no dispersed loci, although presumably some of the rRNA genes are actively expressed (e.g. Figures 2j and 3h). Silver staining of chromosomes at metaphase and nucleoli at interphase is a method to assess activity (Miller *et al.*, 1976). Figure 2c shows that four nucleoli may be present in the cell, and hence all four NORs can be transcriptionally active. Because of nucleolar fusion or inactivity of some NORs (Jordan *et al.*, 1982), many interphases showed only two or three nucleoli in the *A. thaliana* nuclei.

Repetitive centromeric DNA

The probe pAL1 hybridizes to the paracentromeric region of all five chromosome pairs (Figure 3). The position of the hybridization sites (Figure 3b and d) co-localizes with the centromeric block of heterochromatin which stains brightly with DAPI (Figure 3c). The presence of heterochromatin blocks at the centromere has been reported by Ambros and Schweizer (1976), and they are visible at both metaphase and interphase; indeed the chromosome number of *A. thaliana (Stenophragma thalianum)* was first established by Laibach (1907) from counts of chromocentre numbers. Martinez-Zapater *et al.* (1986) suggested that the pAL1, and the related pAS sequences, lie within the heterochromatic blocks seen in *A. thaliana* nuclei. The evidence presented above demonstrates that the sequence is present at all the centromeres, and that all five pairs of chromosomes have a closely similar sequence in the centromeric heterochromatin. Richards *et al.* (1991) have described another genomic clone from *A. thaliana* which is derived from the centromere region of chromosome 1. Like pAL1, it is present as a tandem array, but consists largely of sequences related to that of the true telomere (TTTAGGG), and has perhaps evolved from the pAL1 sequence by insertion of the telomere sequences (Richards *et al.*, 1991), showing that different families of centromeric sequences are capable of independent evolution.

Martinez-Zapater *et al.* (1986) reported various polymorphisms within the pAL1/pAS sequence family. It is unknown whether particular families are more abundant at particular centromeres. However, the polymorphism may be usable to map the positions of centromeres on the RFLP map. The sequence can also be used as a probe on the YAC and cosmid libraries for *A. thaliana* to find the clones containing centromeric DNA.

The rDNA sequence constitutes some 8% of the genome, while the centromeric sequence is under 2%. In the DAPI-stained preparations, each site of centromeric heterochromatin stains with a similar brightness to each rDNA locus, although the length of the sequence is only 10% of that at the rDNA loci (Figure 3g–i). In common with many other species, the *A. thaliana* rDNA is GC rich (AT content 44%; Unfried and Gründler, 1990), while the centromeric sequence, like much heterochromatin, is AT rich (64% AT; Simoens *et al.*, 1988). The preferential binding of DAPI to AT-rich regions of the genome (Schweizer, 1983) probably leads to the observation of similar strengths of fluorescence.

Within the interphase nuclei, the centromeric heterochromatin sites were generally widely distributed over the area of the nucleus (Figure 3e, g and j). This contrasts with the situation in cereals (Anamthawat-Jónsson and Heslop-Harrison, 1990), where centromeres tend to cluster at one pole of the nucleus. The diploid cereal barley has some 20 times the DNA content of *A. thaliana*, much of which is highly or middle repetitive DNA, and almost certainly its presence influences the difference in nuclear architecture.

Nuclear size and ploidy

The interphase nuclei shown vary widely in area and hence presumably volume. Some of the difference may reflect spreading during slide preparation, but wide variation in nuclear size was observed within one slide. Nuclear volume changes with stage of the cell cycle (G1 and G2 nuclei), and chromosome volume is also known to vary by 100% between cells at different stages of development with the same DNA *C* value (Bennett, 1970). However, these factors alone are unlikely to account for the substantial variation observed (cf. Figures 2f and h, and 3e and g), which may result from endopolyploidization or endoreduplication of chromosomes during cell differentiation. Microfluorimetry could be used to confirm such differences before DNA denaturation. Endoreduplication may account for some of the very large hybridization sites observed (e.g. Figure 3f).

The chromosome number in differentiated cells and other non-dividing tissues can vary widely, and is often required in analysis of tissue culture samples, transformed plants and progeny of suspected aneuploids. The pAL1 probe might be useful for determining chromosome number within interphase nuclei because the number of sites of hybridization is readily counted (Figure 3k).

Physical mapping of A. thaliana *chromosomes*

The localization of two repetitive DNA probes on the small chromosomes of *A. thaliana* is a significant improvement in the knowledge of the physical structure of the genome. Not only can the probes be localized at metaphase, but the application of interphase cytogenetic methods, as are used in human studies (Borden and Manuelidis, 1988), is likely to be important in future cytogenetic studies. The

use of multiple probes simultaneously will be useful for physical mapping of low copy sequences on chromosomes where physical markers are unavailable (Leitch *et al.*, 1991). Future work will aim to localize the physical positions of cloned genes and RFLP markers within the *A. thaliana* genome at metaphase and interphase, and hence link genetical, RFLP and physical maps more closely.

Experimental procedures

Plant material

Seeds of *A. thaliana* L. Heynh. cv. Columbia wild-type ($2n = 10$) and trisomic lines originating from M. Koornneef were used. Polyploid lines from the authors' collection (Maluszynska *et al.*, 1990) were also used. The chromosome numbering used in the present paper follows that of Koornneef *et al.* (1983), where chromosome 5 corresponds to chromosome 2 of Schweizer *et al.* (1987), 2 corresponds to 4, and 4 to 5. Chromosomes 1 and 3 are the same in both systems.

Seeds were germinated on Murashige and Skoog (1962) medium with 0.8% agar for 3–4 days before treatment with 2 mM 8-hydroxyquinoline for 30 min at room temperature followed by 30 min at 4°C. They were then fixed in methanol:acetic acid (3:1) for 1–2 h at room temperature and stored at −20°C until use.

Chromosome preparation

Fixed seedlings were washed in 0.01 M citric acid/sodium citrate (pH 4.8) for 15 min and digested in 2% (w/v) cellulase (Calbiochem) and 20% (v/v) pectinase (from *Aspergillus niger*, Sigma, Poole, UK) for 30 min at 37°C (Schwarzacher *et al.*, 1980). The enzyme was carefully washed from the softened material and replaced with buffer for 15–30 min. Root tips were isolated and transferred into 45% acetic acid. A few root tips (3–6) were squashed in a drop of 45% acetic acid onto a glass slide. The quality of chromosome spread was checked under a phase-contrast microscope. Satisfactory preparations were air-dried after removal of the coverslip by freezing.

DNA probes

pTa71. This contains a 9 kb repeat unit of rDNA bounded by *Eco*RI sites, isolated from *Triticum aestivum* (Gerlach and Bedbrook, 1979; recloned and kindly provided by R.B. Flavell and M. O'Dell). The insert contains the 5.8S, 18S, 25S and non-transcribed spacer sequences. The probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) or biotin-11-dUTP (Sigma) by nick translation.

pAL1. Contains a 380 bp tandemly repeated sequence isolated from *A. thaliana* (Martinez-Zapater *et al.*, 1986; kindly provided by C.R. Somerville). The probe was labelled with digoxigenin-11-dUTP using random primer labelling.

For experiments with a single probe, *in-situ* hybridization was carried out using a digoxigenin-anti-digoxigenin-FITC detection technique (Leitch *et al.*, 1991). In the double-probing experiment, pTa71 was labelled with biotin-11-dUTP and pAL1 with digoxigenin as described by Leitch *et al.* (1991). For both *in-situ* hybridizations, labelled probes were mixed to a final concentration of 5 μg ml^{-1} in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS (sodium dodecyl sulphate) and 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) with 0.5 mg ml^{-1} autoclaved salmon sperm DNA.

Hybridization

Slides were treated with 100 μg ml^{-1} DNase-free RNase in 2 × SSC for 1 h at 37°C, washed twice in 2 × SSC for 10 min at room temperature, dehydrated through an ethanol series and air-dried. About 20 μl of hybridization mixture were added to each slide and covered with a plastic coverslip. The chromosomes and DNA probe were denatured together in a humid chamber at 90°C for 10 min. Hybridization was carried out at 37°C in a humid chamber for 12–16 h. After hybridization, coverslips were removed in 2 × SSC at 42°C and then each slide was given a stringent wash for 10 min in 50% (v/v) formamide in 2 × SSC at 42°C, 2 × SSC at 42°C and 2 × SSC at room temperature. The wash removed hybridized sequences with less than 85% identity.

Fluorescent detection and amplification

Sites of probe hybridization were detected with anti-digoxigenin antibody (raised in sheep) conjugated with FITC (fluorescein isothiocyanate) for digoxigenin-labelled DNA or avidin conjugated with Texas Red for biotin-labelled DNA. Slides were transferred to 4 × SSC–Tween buffer (4 × SSC, 0.2% [v/v] Tween 20) for 5 min, treated with 5% (w/v) BSA (bovine serum albumin) in 4 × SSC–Tween for 5 min and then incubated with 20 μl ml^{-1} sheep anti-digoxigenin-FITC, or 20 μg ml^{-1} anti-digoxigenin-FITC and 5 μg ml^{-1} avidin–Texas Red, in 5% BSA for 1 h at 37°C. The slides were washed in 4 × SSC–Tween at 37°C for 3 × 8 min.

For signal amplification, slides were blocked with 5% (v/v) normal goat serum in 4 × SSC–Tween for 5 min and then incubated in 10 μg ml^{-1} rabbit-anti-sheep-FITC, or 25 μg ml^{-1} biotinylated anti-avidin and 10 μg ml^{-1} rabbit-anti-sheep-FITC, in 5% normal goat serum for 1 h at 37°C. The slides were washed in 4 × SSC–Tween 3 × 8 min at 37°C. After washing, double-probed slides were re-incubated in avidin–Texas Red as above.

Slides were counterstained with 2 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) for 15 min and then mounted in antifade solution (AF1, Citifluor). Slides were examined with a Zeiss epifluorescence microscope with filter sets 02, 09 and 12. Photographs were taken on Fujicolor Super HG 400 or Kodak Ektar 1000 colour print film.

Silver staining

Slides were incubated for 10 min in 0.01 M borate buffer (pH 9.2). A few drops of freshly prepared 50% silver nitrate solution in distilled water were applied to the preparation (after Bloom and Goodpasture, 1976). Slides were covered with a nylon mesh (Hizume *et al.*, 1980), incubated in a moist chamber at 60°C for 30 min, washed in distilled water and air-dried.

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References

Ambros, P. and Schweizer, D. (1976) The Giemsa C-banded karyotype of *Arabidopsis thaliana*. Arabidopsis *Info. Service*, **13**, 167–171.

Anamthawat-Jónsson, K. and Heslop-Harrison, J.S. (1990) Centromeres, telomeres and chromatin in the interphase nucleus of cereals. *Caryologia*, **43**, 205–213.

Bennett, M.D. (1970) Natural variation in nuclear characters of meristems of *Vicia faba*. *Chromosoma*, **29**, 317–335.

Bennett, M.D. and Smith, J.B. (1976) Nuclear DNA amount in angiosperms. *Phil. Trans. R. Soc. B*, **274**, 227–274.

Bennett, M.D., Smith, J.B. and Heslop-Harrison, J.S. (1982) Nuclear DNA amounts in angiosperms. *Proc. R. Soc. Lond. B*, **216**, 179–199.

Bloom, S.E. and Goodpasture, C. (1976) An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. *Hum. Genet.* **34**, 199–206.

Borden, J. and Manuelidis, L. (1988) Movement of the X chromosome in epilepsy. *Science*, **242**, 1687–1691.

Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S. and Meyerowitz, E.M. (1988) Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **85**, 6856–6860.

Disteche, C.M., McConnell, G.K., Grant, S.G., Stephenson, D.A., Chapman, V.M., Gandy, S. and Adler, D.A. (1989) Comparison of the physical and recombination maps of the mouse X chromosome. *Genomics*, **5**, 177–184.

Flavell, R.B. and Smith, D.B. (1974) Variation in nucleolar organizer rDNA gene multiplicity in wheat and rye. *Chromosoma*, **47**, 327–334.

Gerlach, W.L. and Bedbrook, J.R. (1979) Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucl. Acids Res.* **7**, 1869–1885.

Gustafson, J.P., Butler, E. and McIntyre, C.L. (1990) Physical mapping of a low-copy DNA sequence in rye (*Secale cereale* L.). *Proc. Natl Acad. Sci. USA*, **87**, 1899–1902.

Heslop-Harrison, J.S. (1991) The molecular cytogenetics of plants. *J. Cell Sci*. **100**, 1–7.

Hizume, M., Sato, S. and Tanaka, A. (1980) A highly reproducible method of nucleolus organizer region staining in plants. *Stain Technol.* **55**, 87–90.

Jordan, E.G., Martini, G. and Bennett, M.D. (1982) Nucleolar fusion in wheat. *J. Cell Sci*. **56**, 486–495.

Koornneef, M., van Eden, J., Hanhart, C.J., Braaksma, F.J. and Feenstra, W.J. (1983) Linkage map of *Arabidopsis thaliana*. *J. Hered.* **74**, 265–272.

Laibach, F. (1907) Zur Frage nach der Individualität der Chromosomen in Pflanzenreich. *Beih. Bot. Cbl.* 1 Abt. **22**, 191–210.

Leitch, I.J., Leitch, A.R. and Heslop-Harrison, J.S. (1991) Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. *Genome*, **34**, 329–333.

Leutwiler, L.S., Hough-Evans, B.R. and Meyerowitz, E.M. (1984) The DNA of *Arabidopsis thaliana*. *Mol. Gen. Genet.* **194**, 15–23.

Maluszynska, J. and Schweizer, D. (1989) Ribosomal RNA genes in B chromosomes of *Crepis capillaris* detected by non-radioactive *in situ* hybridization. *Heredity*, **62**, 59–65.

Maluszynska, J., Maluszynski, M., Rebes, G. and Wietrzyk, E. (1990) Induced polyploids of *Arabidopsis thaliana*. In *Abstracts of the Fourth International Conference on* Arabidopsis *Research* (Schweizer, D., Peuker, K. and Loidl, J., eds). Vienna: University of Vienna, p.4.

Martinez-Zapater, J.M., Estelle, M.A. and Somerville, C.R. (1986) A highly repeated DNA sequence in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **204**, 417–423.

Meyerowitz, E.M. and Pruitt, R.E. (1985) *Arabidopsis thaliana* and plant molecular genetics. *Science*, **229**, 1214–1218.

Miller, D.A., Dev, V.G., Tantravahi, R. and Miller, O.J. (1976) Suppression of human nucleolus organiser activity in mouse-human somatic hybrid cells. *Exp. Cell Res.* **101**, 235–243.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 479–497.

Murata, M., Varga, F., Maluszynska, J., Gründler, P. and Schweizer, D. (1990) Chromosomal localization of the ribosomal RNA genes in *Arabidopsis thaliana* by *in situ* hybridization. In: *Abstracts of the Fourth International Conference on* Arabidopsis *Research* (Schweizer, D., Peuker, K. and Loidl, J., eds). Vienna: University of Vienna, p. 5.

Nam, H.G., Giraudat, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M. and Goodman, H.M. (1989) Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana*. *Plant Cell*, **1**, 699–705.

Pruitt, R.E. and Meyerowitz, E.M. (1986) Characterization of the genome of *Arabidopsis thaliana*. *J. Mol. Biol.* **187**, 169–183.

Richards, E.J., Giraudat, J., Goodman, H.M. and Ausubel, F.M. (1991) The centromere region of *Arabidopsis thaliana* chromosome 1 contains telomere-similar sequences. *Nucl. Acids Res.* in press.

Schwarzacher, H.G. and Wachtler, F. (1983) Nucleolus organizer regions and nucleoli. *Hum. Genet.* **63**, 89–99.

Schwarzacher, T. and Heslop-Harrison, J.S. (1991) *In situ* hybridization to plant telomeres using synthetic oligomers. *Genome*, **34**, 317–323.

Schwarzacher, T., Ambros, P. and Schweizer, D. (1980) Application of Giemsa banding to orchid karyotype analysis. *Plant Syst. Evol.* **134**, 293–297.

Schweizer, D. (1983) Distamycin-DAPI bands: properties and occurrence in species. In: *Kew Chromosome Conference II* (Brandham, P.E. and Bennett, M.D., eds). London: George Allen and Unwin, pp. 43–51.

Schweizer, D., Ambros, P., Gründler, P. and Varga, F. (1987) Attempts to relate cytological and molecular chromosome data of *Arabidopsis thaliana* to its genetic linkage map. Arabidopsis *Info. Service*, **25**, 27–35.

Sears, L.M.S. and Lee-Chen, S. (1970) Cytogenetic studies in *Arabidopsis thaliana*. *Can. J. Genet. Cytol.* **12**, 217–223.

Simoens, C.R., Gielen, J., Van Montagu, M. and Inze, D. (1988) Characterization of highly repetitive sequences of *Arabidopsis thaliana*. *Nucl. Acids Res.* **16**, 6753–6766.

Snape, J.W., Flavell, R.B., O'Dell, M., Hughes, W.G. and Payne, P.I. (1985) Intrachromosomal mapping of the nucleolar organizer region relative to three marker loci on chromosome 1B of wheat (*Triticum aestivum*). *Theor. Appl. Genet.* **69**, 263–270.

Steinitz-Sears, L.M. (1963) Chromosome studies in *Arabidopsis thaliana*. *Genetics*, **48**, 483–490.

Unfried, I. and Gründler, P. (1990) Nucleotide sequence of the 5.8S and 25S rRNA genes and the internal transcribed spacers from *Arabidopsis thaliana*. *Nucl. Acids Res.* **18**, 4011.

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Imaging of DNA in human and plant chromosomes by high-resolution scanning electron microscopy

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We describe a DNA-specific staining procedure, using a blue platinum organic dye, which allows DNA imaging of chromosomes by detection of back-scattered electrons in the scanning electron microscope. DNA distribution is visualized within chromosomal details of the centromeric and satellite regions, or in interphase chromatin, with high-resolution (10–15 nm) for comparison with the corresponding secondary electron image representing DNA plus protein.

Key words: chromosomes, DNA staining, EDX, platinum blue, scanning electron microscopy

Introduction

Chromosomes were first detected by Flemming and were so named by Waldeyer in 1888 because of their ability to adsorb basic dyes. While visualization of chromosomes by bright-field light microscopy has improved since then, the classical dyeing techniques are still in use. Phase-contrast microscopy was an important development, permitting studies of chromosome movement in living cells. Further progress has been made with the introduction of interference-contrast techniques because the low depth of field of this technique allows focusing through cells with high resolution. An important area of chromosome research is now the application of fluorescence microscopy.

What is really visualized by these methods? Alkaline dyes and fluorescence microscopy provide an image of DNA distribution. The contrast of metaphase chromosomes in phase-contrast microscopy arises from the high concentration of DNA and its high refractive index. Interference-contrast microscopy, which plays a minor role, gives an image of both the DNA and protein, which results, for example, in less pronounced primary and secondary constrictions (Wanner *et al.* 1991). Therefore our structural models of chromatin are mainly based on DNA imaging.

In conventional transmission electron microscopy of ultra-thin sections, fixed with glutaraldehyde/osmium and post-stained with lead citrate and uranyl acetate, it is difficult to distinguish between DNA and protein. This is especially true when structural elements such as solenoids and supersolenoids are superimposed in thick sections (50–70 nm).

The three-dimensional structure of chromosomes, elucidated by scanning electron microscopic studies by several groups, has been well documented (Harrison *et al.* 1982, 1987, Mullinger & Johnson 1987, Rattner 1987, Sumner, 1991, Wanner *et al.* 1991, Pelling & Allen 1993, Takayama & Hiramatsu 1993, Rizzoli *et al.* 1994). The secondary electron signal, however, does not distinguish between the protein and DNA components. Consequently, the use of the scanning electron microscopy to confirm any of the structures postulated in competing chromosome models (radial loops, folded fibres, supersolenoid, scaffold, etc.) is of limited value. Therefore, a method which specifically stains only DNA for scanning electron microscopy is of great importance.

Materials and methods

Potassium tetrachloroplatinate (2 g) was mixed with acetonitrile (3 ml) in water (40 ml) at room temperature. After approximately 10 days, precipitation of pale yellow crystals of platinum dichlorodiacetamide was complete. After decanting the liquid, this crystalline complex was air dried and shaken with the same weight of silver sulphate and a fivefold volume of water. After several hours, when the blue colour had reached maximum intensity, indicating complete conversion to platinum blue, a 10-fold volume of methanol was added. The solution was then filtered before precipitating the platinum blue with diethyl ether. The dye was collected by filtration and air dried (Hofmann & Bugge 1908).

Chromosomes were prepared as described by Martin *et al.* (1994). For comparison, chromosomes were fixed with 2.5% glutaraldehyde in 75 mM cacodylate buffer

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and then incubated at room temperature, unless otherwise specified, for:

(a) 30 min with 10 mM platinum blue in 50 mM Tris buffer containing 2 mM $MgCl_2$ at pH 7.5.
(b) 30 min with 10 μg/ml DNAase at 37°C followed by platinum blue treatment (step a above).
(c) 30 min with 1% osmium tetroxide in water.
(d) 30 min with 1% uranyl acetate in water.
(e) 10 min with 1% osmium tetroxide (Os) and 10 min with saturated thiocarbohydrazide (TCH) in the four orders and combinations specified below:
    (1) Os, TCH
    (2) TCH, Os
    (3) Os, TCH, Os
    (4) TCH, Os, TCH, Os
(f) 10 or 30 min with buffer only (control).

After the above treatments, the chromosome preparations were washed three times with water, dehydrated with a graded series of acetone concentrations and critical point dried from liquid CO_2. The specimens were coated with about 3 nm of carbon by evaporation. All specimens were examined with a Hitachi S-4100 field-emission scanning electron microscope operated at various voltages. Back-scattered electrons (BSEs) were monitored at 15 kV with an Autrata detector of the YAG type.

Element analysis was performed with a Tracor 'Voyager' system equipped with a light element silicon detector (Pioneer) and an ultra-thin window (tilting angle of 30°, working distance 15 mm, maximum peak counts set to 2000). As the weak osmium signal is close to the large silicon peak of the glass slide, chromosomes were also prepared on plastic slides made from Melinex (polyethylene terephthalate) sheets.

Results

Comparison of metal impregnations

Most biological specimens are electrical insulators. If they have an elaborate surface topography they must be coated with metal to avoid charging effects, at a thickness that does not obscure fine structural detail. Metal impregnation is known to increase specimen conductivity. For chromosome impregnation, the osmium–TCH–osmium technique, first described by Ip & Fischman (1979) for cytoskeletal elements, is commonly used (Harrison *et al.* 1982, Allen *et al.* 1986, Sumner 1991).

When human or plant chromosomes were fixed only with glutaraldehyde, the BSE signal was very weak, so that the chromosomes could barely be detected at low magnification (Figure 1a). Impregnation of chromosomes with osmium should give a strong BSE signal. Surprisingly, impregnation of plant and human chromosomes with osmium or osmium TCH (see Materials and methods section) gave only a minor enhancement of contrast (Figure 1b), which was not increased by a second osmium treatment cycle. In a few osmium-impregnated preparations of human chromosomes, reasonable contrast was observed, as reported by several other authors (Sumner 1991, Rizzoli *et al.* 1994). However, as preparation of human chromosomes improved, impregnation by osmium was considerably reduced, and impregnation of plant chromosomes has never been observed. We infer that the earlier observations of impregnation of human chromosomes were due to the presence of osmiophilic (karyoplasmic?) impurities.

Impregnation of both human and plant chromosomes with uranyl acetate gave a BSE image with good contrast (Figure 1c). The best contrast of chromosomes, however, was achieved after impregnation with platinum blue (Figure 1d).

These data are in good agreement with the energy dispersive X-ray analysis (EDX) spectra (Figure 2). Chromosomal composition is best visualized at 5 kV as the low-energy beam does not reach the glass support. In this case, even nitrogen is resolved. Unfortunately, uranium cannot be monitored at 5 kV. Consequently, for comparison of the different metal impregnation procedures, the spectra presented were taken at both 5 kV and 10 kV. The control (chromosomes fixed only with gutaraldehyde) shows four peaks characteristic of chromosomes: carbon, oxygen, sodium and phosphorus (Figure 2B, b). The other peaks derive from the glass slide (Figure 2A, a): sodium is present both in the slide and as the counter-ion to the phosphodiester group in DNA.

The EDX spectra of osmium-impregnated chromosomes (Figure 2C, c) confirm the weak reaction: the osmium peak causes only a broadening of the phosphorus peak. As the osmium peak is located close to the silicon peak of the glass slide, chromosomes were also prepared on plastic slides made from Melinex (polyethylene terephthalate) sheets. The EDX analysis for osmium-treated preparations was improved in resolution but confirmed the very weak incorporation. Both uranyl acetate (Figure 2D) and platinum blue (Figure 2E, e) are easily detected by EDX; however, as already seen in the BSE images, the platinum signal is much stronger than that of uranium. The enhancement of the nitrogen signal is due to the nitrogen content of platinum blue. DNAase-treated chromosomes were stained only weakly, if at all, with platinum blue (Figure 2F, f). Owing to mass loss (reduction of carbon, nitrogen, phosphorus and platinum peaks), the beam penetrates into the glass slide, causing enhancement of the oxygen and silicon peaks.

Specific staining of DNA in chromosomes

Platinum blue forms oligomers: the molecular weight of the monomer is 311. The dye is readily soluble in water to give a concentration of 20 mM and a pH of 9. As a solid, or in solution, it is stable at room temperature for at least 1 month. Platinum blue and related platinum

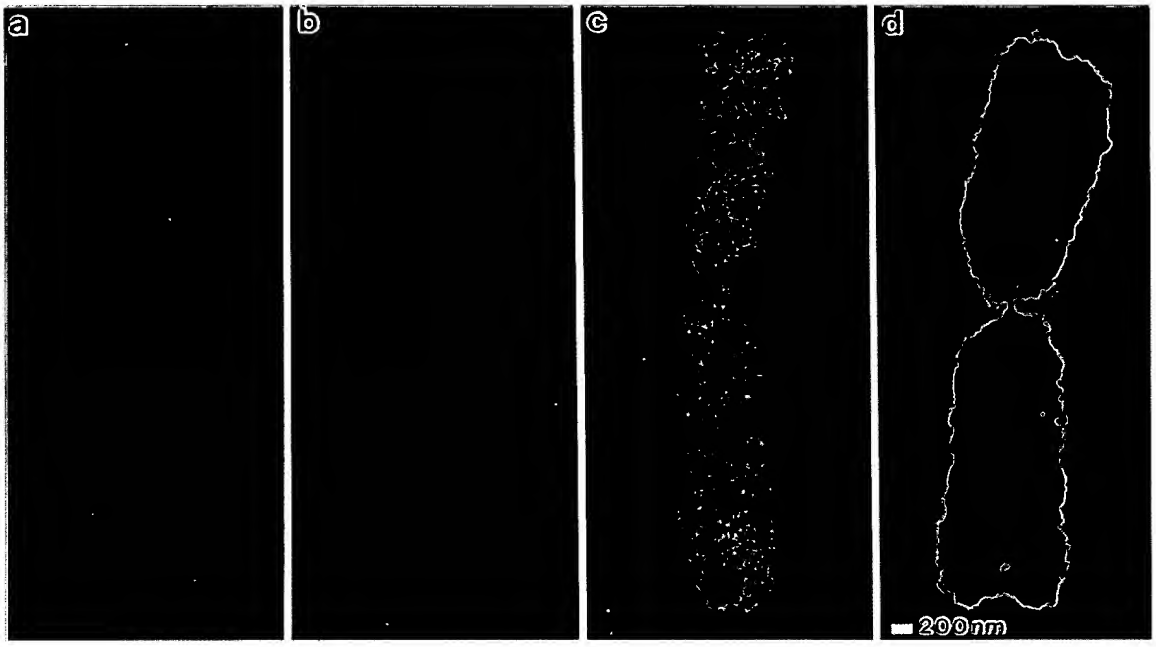

Figure 1. Comparison of BSE images of barley metaphase chromosomes stained with different compounds: **a** control (glutaraldehyde only); **b** osmium tetroxide; **c** uranyl acetate; **d** platinum blue. Only uranyl acetate and platinum blue gave sufficient contrast.

organic compounds selectively react with nucleic acids, especially with DNA (Aggarwal *et al.* 1975, 1977, Lippert & Beck 1983, Köpf-Maier & Köpf 1986, Frommer *et al.* 1990). Large chromosomes, such as those from the genus *Lilium*, incorporate so much platinum blue that they appear pale blue in the light microscope; however, there is no significant change in image characteristics as shown by observation with phase-contrast, interference-contrast, epifluorescence or epipolarization microscopy.

When viewing platinum blue-stained chromosomal preparations by BSE microscopy at low magnification, the nuclei and chromosomes are clearly visible and appear very bright at all stages of condensation. Interphase nuclei exhibit a fibrous network mixed with nodular elements. The nucleolus and heterochromatic regions appear much brighter than euchromatin. During all stages of condensation from early prophase to telophase, the chromosomes are easily monitored by the electron microscope in the BSE mode; in secondary electron (SE) mode, however, the chromosomes are only detected when liberated from karyoplasmic material. Even when chromosomes are embedded in a dense proteinaceous network, good contrast makes all their characteristic details clearly visible, including chromatids and primary and secondary constrictions (Figures 3–6).

When superimposing chromosome images, a good fit is generally obtained between the BSE and SE images, although the chromosomes appear smaller in diameter in the BSE image. The most striking difference between the images occurs in the centromeric and satellite region: both are frequently much thinner and darker than the chromatids and often appear as a black gap. Higher magnification reveals that thin filaments are indeed present (Figures 4 & 5).

Observing the SE image of chromosomes, the chromatids only become visible at late metaphase when their separation begins (x-form). In the BSE mode, chromatid separation can already be seen in early metaphase (Figure 3).

When using a test specimen (colloidal gold on collodion films), the resolution of the Autrata BSE detector is better than 5 nm. Owing to chromosome thickness (0.5–1 μm) and beam penetration (about 3 μm) in a carbon-coated specimen at 15 kV, the resolution of chromosomal detail in the BSE mode is limited mainly by superimposition and scattering effects. When investigating flattened prophase chromosomes, the thinnest fibres which can be resolved range between 10 and 15 nm (Figure 6). At high magnification, the DNA specificity of the platinum blue is still striking: even at the resolution limit, DNA-containing (stained) and proteinaceous elements (unstained) can be easily distinguished.

Superimposition effects of structural elements within

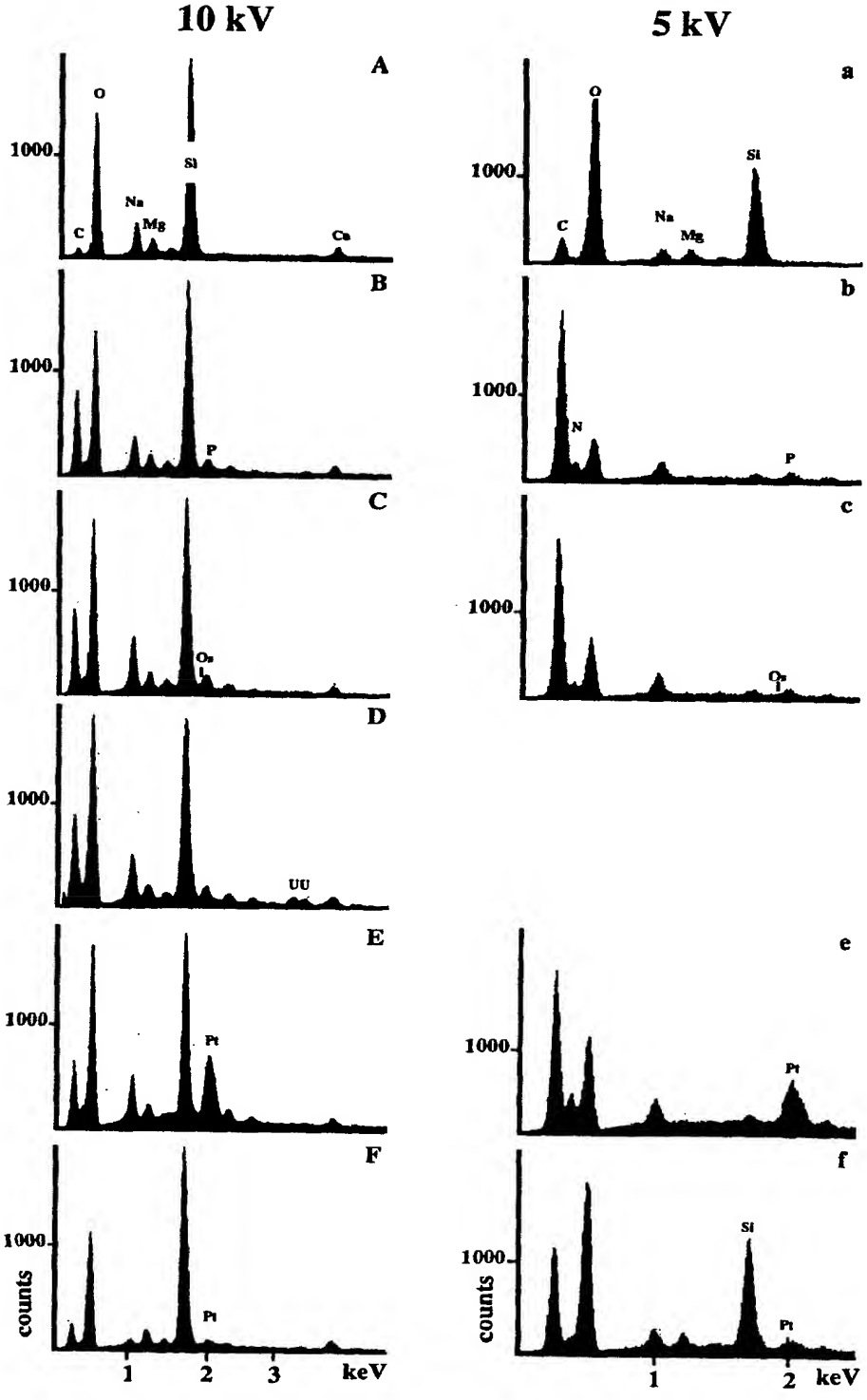

Figure 2. Comparison of EDX spectra at acceleration voltages of 10 kV (**A–F**) and 5 kV (**a–f**) of barley metaphase chromosomes treated as follows: **A**, **a**, glass slide; **B**, **b**, control (untreated); **C**, **c**, osmium tetroxide; **D**, **d**, uranyl acetate; **E**, **e**, platinum blue; **F**, **f**, DNAase-treated chromosomes, platinum blue stained. Spectrum **d** is not shown because the beam energy is too low to detect uranium.

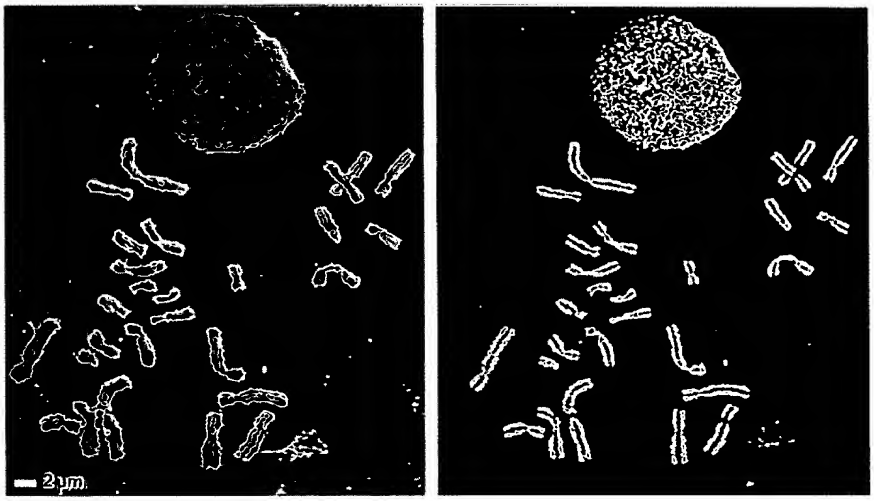

Figure 3. Comparison of SE and BSE images of human metaphase chromosomes stained with DNA-specific platinum blue. Even at a low magnification the contrast between the chromosomes and the dark background is striking. Chromatid separation is already visible in the BSE image. An interphase, the nucleus shows euchromatic and heterochromatic regions. Left side: SE image; right side: BSE image. The specimen was only carbon coated.

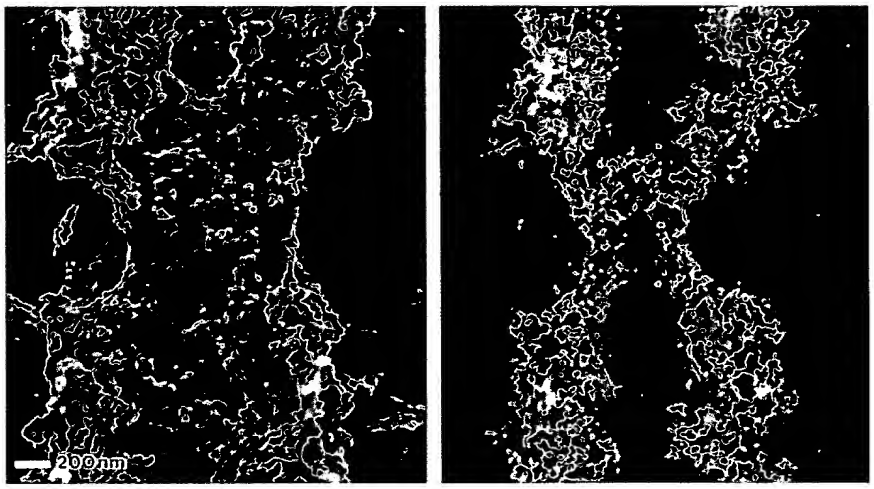

Figure 4. Centromeric region of a human metaphase chromosome stained with DNA-specific platinum blue. The BSE image (DNA) exhibits a fibrous network which is less compact than the structure observed in the SE mode (DNA + protein). Left side: SE image; right side: BSE image. The specimen was only carbon coated.

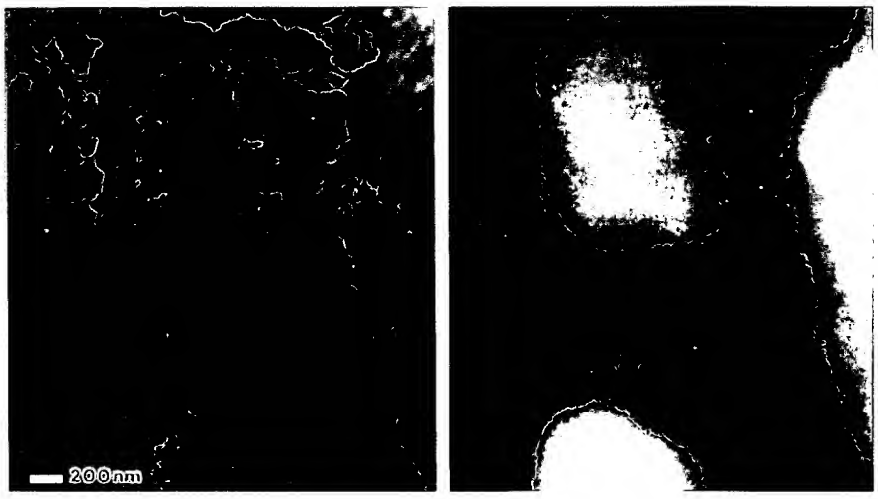

Figure 5. Satellite region of a barley metaphase chromosome stained with DNA-specific platinum blue. The secondary constriction appears as a rather solid element in the SE image but the BSE image reveals that only a few fibres (DNA-containing elements) are involved in a loose network. Left side: SE image; right side: BSE image. The specimen was only carbon coated.

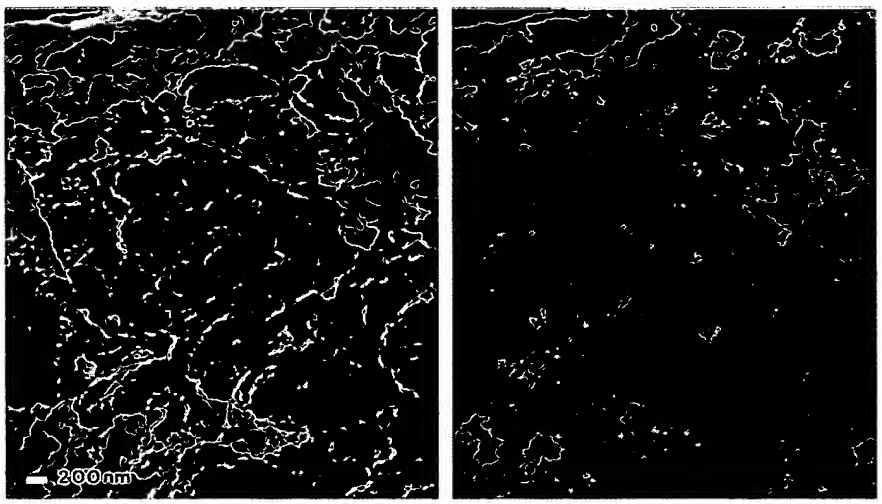

Figure 6. Interphase chromatin of a human nucleus stained with platinum blue. The BSE image proves the specificity and resolution of the DNA-specific dye for scanning electron microscopy. Regions of higher DNA concentration are brightly visible; the thinnest fibres that can be resolved in the BSE image range between 10 nm and 20 nm. Left side: SE image; right side: BSE image. The specimen was only carbon coated.

the chromosome, typical of BSE images, severely limit interpretation of complex three-dimensional architectures; however, stereopairs of both SE and BSE images can provide an excellent insight into the three-dimensional architecture of chromosomes.

Discussion

There are a variety of platino-organic compounds with blue colour (Lippert, 1988). Because they interact with both DNA and RNA these dyes are used as specific stains for transmission electron microscopy (Aggarwal *et al.* 1975, 1977) and are potential candidates for cell growth inhibition, especially inhibition of tumour growth (Lippert & Beck, 1983, Köpf-Maier & Köpf 1986). The wide pH range (pH 3–9) of the specific reaction between platinum blue and nucleic acids, determined by precipitation makes it ideal for scanning electron microscopic studies of chromosomes under both stabilizing and denaturing conditions.

The high yield of both SE and BSE has two effects: although coated only with carbon, the topography of the SE image is quite good and the specific binding to nucleic acids gives a very strong BSE signal for mapping both DNA concentration and distribution. As the depth of origin of the BSE is well within the range of most chromosome diameters, it provides the possibility of combining three-dimensional topography with the spatial arrangement of DNA. The apparent lack of DNA in primary and secondary constrictions as seen by epifluorescence microscopy corresponds to a weak BSE signal but is not correlated with a similar constriction of total chromosome structure in the SE image. Consequently, DNA staining with organic platinum compounds will be a powerful tool for investigating DNA in many chromosomal aberrations, including deletions, radiation damage sites and cold segments; concurrently, information may be obtained about possible damage to or stability of proteinaceous and structural elements such as scaffolds.

More work should be done on chromosome staining with various platinum compounds because there are some compounds for which GC specificity has been demonstrated (Dieter-Wurm *et al.* 1992): It would be of great interest, for example, to determine if banding of intact (undigested) chromosomes is possible and if it correlates with the three-dimensional structure of the chromosome.

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References

Aggarwal SK, Wagner RW, McAllister PK, Rosenberg B (1975) Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum–pyrimidine complexes by electron microscopy. *Proc Natl Acad Sci USA* 72: 928–932.

Aggarwal SK, Ofosu GA, Waku Y (1977) Cytotoxic effects of platinum–pyrimidine complexes on the mammalian cell in vitro: a fine structural study. *J Clin Hematol Oncol* 7: 547–561.

Allen TD, Jack EM, Harrison CJ, Claugher D, Harris R (1986) Human metaphase chromosome preparation for scanning

electron microscopy – a consideration of inherent problems. In: O'Hare AMF ed. *Science of Biological Specimen Preparation*. Chicago: SEM, pp 299–307.

Dieter-Wurm I. Sabat M, Lippert B (1992) Model of platinated DNA-triplex: Watson–Crick and metal modified Hoogsteen pairing. *J Am Chem Soc* **144**: 357–359.

Frommer G, Schöllhorn H, Thewalt U, Lippert B (1990) Platinum(II) binding to N7 and N1 of guanine and a model for a purine-N^1,Pyrimidine-N^3 cross-link of cisplatin in the interior of a DNA duplex. *Inorganic Chem* **29**: 1417–1422.

Harrison CJ, Britch M, Allen TD, Harris R (1982) High resolution SEM of human metaphase chromosomes. *J Cell Sci* **56**: 409–422.

Harrison CJ, Jack EM, Allen TD (1987) Light- and scanning electron microscopy of the same metaphase chromosomes. In: Hayat MA, ed. *Correlative Microscopy in Biology: Instrumentation and Methods*. New York: Academic Press, pp 187–248.

Hofmann KA and Bugge G (1908) Platinblau. *Ber Chem Ges* **41**: 312.

Ip W, Fischman DA (1979) High resolution scanning electron microscopy of isolated in situ cytoskeletal elements. *J Cell Biol* **83**: 249–253.

Köpf-Maier P, Köpf H (1986) Cytostatische Platin-Komplexe: eine unerwartete Entdeckung mit weitreichenden Konsequenzen. *Naturwissenschaften* **73**: 239–247.

Lippert B (1988) Platinum nucleobase chemistry. *Prog Inorganic Chem* **17**: 1–97.

Lippert B, Beck W (1983) Platinkomplexe in der Krebstherapie. *Chem u Z* **17**: 190–197.

Martin R, Busch W, Herrmann RG, Wanner G (1994) Efficient preparation of plant chromosomes for high-resolution scanning electron microscopy. *Chrom Res* **2**: 411–415.

Mullinger AM, Johnson RT (1987) Scanning electron microscope analysis of structural changes and aberrations in human chromosomes associated with the inhibition and reversal of inhibition of ultraviolet light induced DNA repair. *Chromosoma* **96**: 39–44.

Pelling C, Allen TD (1993) Scanning electron microscopy of polytene chromosomes (I). *Chrom Res* **1**: 221–237.

Rattner JB (1987) The organization of the mammalian kinetochore: a scanning electron microscope study. *Chromosoma* **95**: 175–181.

Rizzoli R, Rizzi E, Falconi M *et al* (1994) High resolution detection of uncoated metaphase chromosomes by means of field emission scanning electron microscopy. *Chromosoma* **103**: 393–400.

Sumner AT (1991) Scanning electron microscopy of mammalian chromosomes from prophase to telophase, *Chromosoma* **100**: 410–418.

Wanner G, Formanek H, Martin R, Herrmann RG (1991) High resolution scanning electron microscopy of plant chromosomes. *Chromosoma* **100**: 103–109.

Takayama S, Hiramatsu H (1993) Scanning electron microscopy of the centromeric region of L-cell chromosomes after treatment with Hoechst 33258 combined with 5-bromodeoxyuridine. *Chromosoma* **102**: 227–232.

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ORIGINAL PAPER

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Physical mapping of the 5S ribosomal RNA genes on rice chromosome 11

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Abstract One 5S ribosomal RNA gene (5S rDNA) locus was localized on chromosome 11 of japonica rice by *in situ* hybridization. The biotinylated DNA probe used was prepared by direct cloning and direct labeling methods, and the locus was localized to the proximal region of the short arm of chromosome 11 (11p1.1) by imaging methods. The distance between the signal site and the centromere is 4.0 arbitrary units, where the total length of the short arm is 43.3 units. The 5S rDNA locus physically identified and mapped in rice was designated as *5SRrn*. The position of the 5S rDNA locus reported here differs from that in indica rice; possible reasons for this difference are discussed. DNA sequences of 5S rDNA are also reported.

Key words Rice · 5S ribosomal RNA gene · *In situ* hybridization · Imaging · 5S rDNA sequence

Introduction

In situ hybridization (ISH) is an effective method for physical mapping of specific RNA and/or DNA sequences on chromosomes (Fukui 1984). There have been several reports on the physical mapping of the rice (*Oryza sativa*) 17S, 5.8S and 25S ribosomal RNA genes (rDNA) using ISH with radioactive, ^{125}I-labeled rRNA probes (Fukui et al. 1987) or with biotin-labeled probes (Fukui 1990; Islam-Faridi et al. 1990; Iijima et al. 1991). One rDNA locus has been physically mapped at the end of the short arm of chromosome 9 by ISH (Iijima et al. 1991; Fukui and Iijima 1992) in the course of the development of a quantitative chromosome map of japonica rice (Fukui and Iijima 1991), whereas two rDNA loci have been mapped at the termini of the short arms of chromosomes 9 and 10 of indica rice (Islam-Faridi et al. 1990; Fukui et al. 1994b).

Another ribosomal RNA gene, the 5S rRNA gene (5S rDNA), has been detected at a position distinct from that of the major rDNA loci in wheat (Mukai et al. 1990), tomato (Lapitan et al. 1991), and barley (Leitch and Heslop-Harrison 1993; Fukui et al. 1994a). Song and Gustafson (1993) have recently reported the localization of the 5S rDNA locus at the end of the short arm of chromosome 9, the site at which the other ribosomal RNA genes are also located, in indica rice.

We report here the mapping of the rice 5S rDNA locus in japonica rice by an improved ISH method, and nucleotide sequencing of the gene. We demonstrate the effectiveness of the ISH method in conjunction with imaging methods for the analysis of chromosome organization in rice. The results obtained differ from those for indica rice previously reported by Song and Gustafson (1993).

Materials and methods

Plant material

'Nipponbare', a japonica rice (*Oryza sativa* L.) variety, was used as the plant material for both chromosome preparations and total DNA extraction. Root tips about 1 cm long for chromosome preparation were collected from kernels germinated at 32° C. Total

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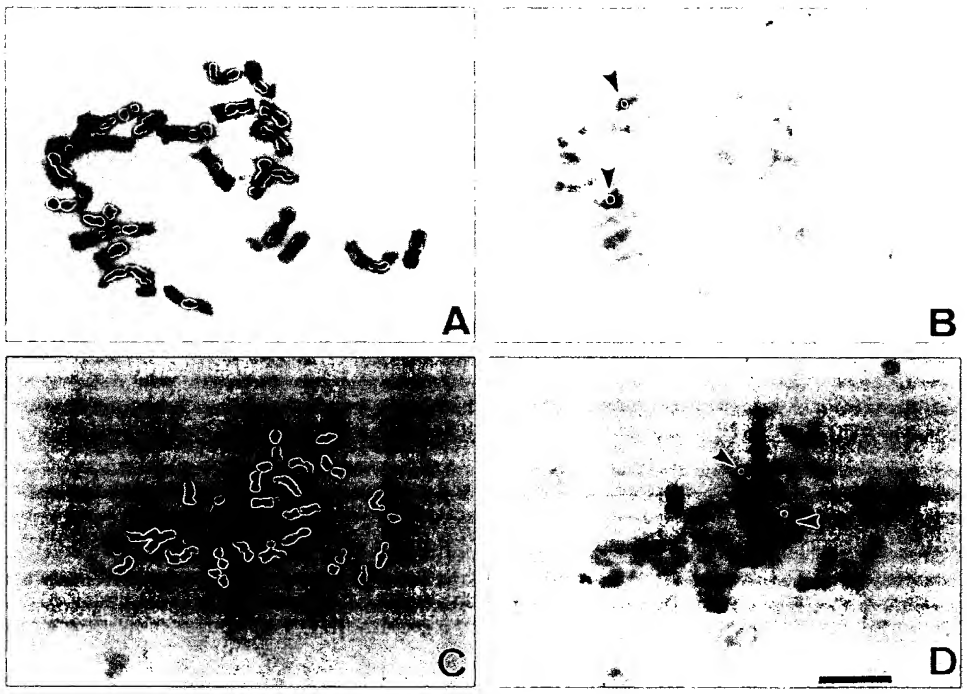


Fig. 1A–D Rice chromosomes before and after ISH. A, C Giemsa-stained rice chromosomes. Chromosomes 11 are indicated by *arrowheads*. B, D Chromosome spreads after ISH with probe for 5S rDNA. Bar, 5 μm

genomic DNA was extracted from the leaf tissues of the seedlings of Nipponbare by the cetyltrimethyl ammonium bromide method.

Chromosome sample preparation

The enzyme maceration/air drying method was used throughout for the preparation of the chromosome samples. Details of the method have previously been described (Fukui and Iijima 1991, 1992; Iijima and Fukui 1991). Good chromosomal spreads that showed a clear condensation pattern (CP; Fukui and Mukai 1988) allowing identification of each chromosome were surveyed and photographed. All the rice chromosomes in the spreads were visually identified by their CP prior to ISH.

In situ hybridization (ISH)

ISH was performed by the method already described (Iijima et al. 1991; Fukui and Iijima 1992). The biotinylated 5S rDNA was prepared from interphase nuclei of Nipponbare by the direct-cloning and direct-labeling methods developed by Fukui et al. (1994a). Briefly, fixed root tips were enzymatically macerated on polyester membrane disks. Fragments bearing about 100 nuclei on cut out and transferred individually to 500-μl microtubes, treated with a Proteinase K solution (1 mg/ml; Wako Pure Chemical Industries, Osaka) and subjected to PCR with 70% substitution of biotin-11-dUTP for dTTP (Sigma).

Image analysis of the ISH signals

Chromosome spreads with signals were photographed. The enlarged microphotographs were scanned using the Chromosome image analyzing system II (CHIAS2/VIDAS Zeiss/Kontron). After standard image processing for noise reduction, contrast enhancement, enlargement etc. were performed as described previously (Fukui 1985; 1986; 1988; Fukui and Kamisugi 1991a, b; Kamisugi and Fukui 1992), the signals were pseudo-colored or pseudo-three dimensionally processed using the enlarged gray image of the chromosomes. Pseudo-coloration and pseudo-three dimensional construction of the chromosome images were performed as described in detail by Fukui and Ito (1989) and Fukui and Kamisugi (1991a, b).

Lengths of the short and long arms, and the section length from the center of the signal to the centromere of the chromatid were digitally measured. 5S rDNA was then located on the standard quantitative rice chromosome map established by imaging methods (Fukui and Iijima 1991, 1992).

Cloning and DNA sequencing of the 5S rDNA fragments

To clone the DNA fragments containing 5S rDNA, PCR was carried out under the conditions described previously (Mochizuku et al. 1992) using 2.5 units of *Taq* DNA polymerase (Promega), 0.5 μg total DNA from Nipponbare, and 1 μM each of a pair of primers, 5′-GATCCCATCAGAACTCC-3′ and 5′-GGTGCTTTAGTGCTGGTAT-3′, which hybridized to the two strands of the 5S rRNA coding sequence. The amplified fragments were ligated to a linear pCRII vector using a TA cloning kit (Invitrogen) and introduced by transformation into InVαF cells (Invitrogen). DNA sequencing was carried out by the dideoxynucleotide chain termination method (Messing 1983; Sanger et al. 1977) using Sequenase Ver. 2.0 (United States Biochemicals.)

Results

Figure 1 shows two rice chromosome complements of Nipponbare before (Fig. 1A, C) and after ISH

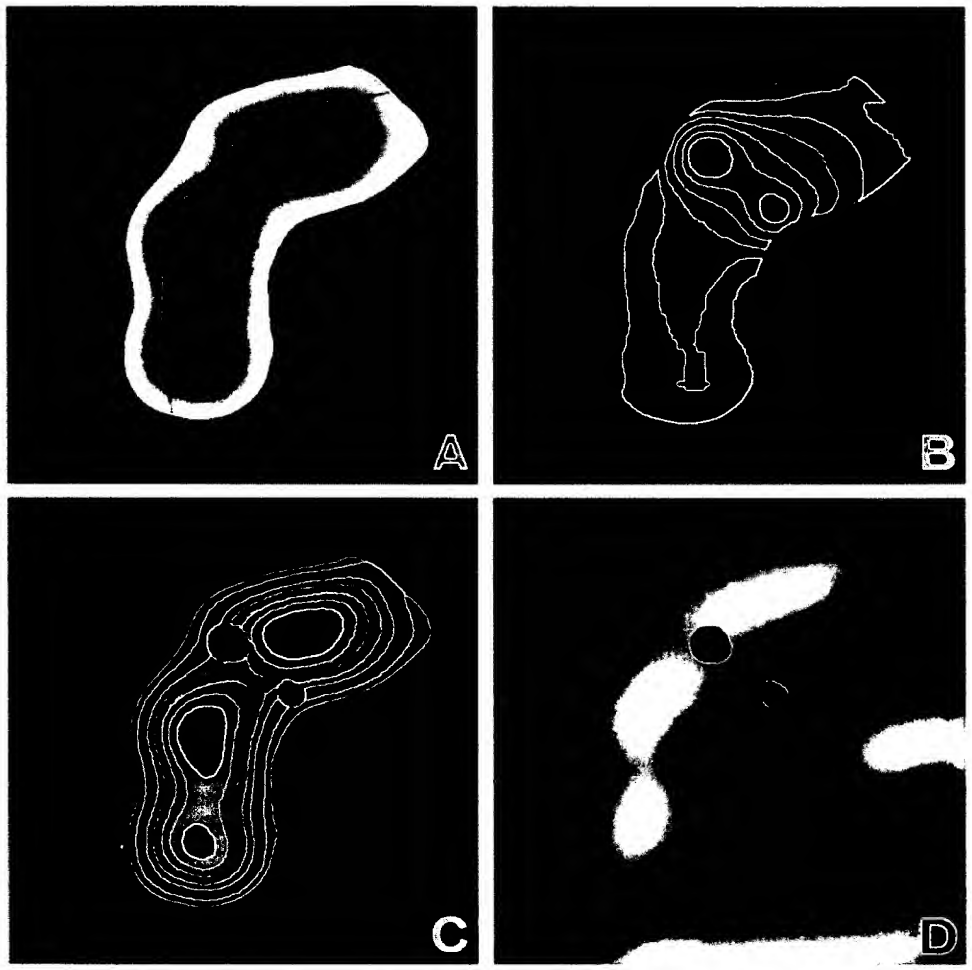


Fig. 2A–D Imaging of rice chromosome 11 before and after ISH. A Giemsa-stained original image and C its pseudo-colored representation with signals superimposed. B Signal image after pseudo-coloring and (D) in a pseudo-three dimensional representation

(Fig. 1B, D). Clear condensation patterns observed for each of the Giemsa-stained rice chromosomes (Fig. 1A, C) enabled us to identify the chromosomes individually.

Figures 1B and 1D show the two signals in each of the complements. Comparison of the photographs before (Fig. 1A, C) and after ISH (Fig. 1B, D) allows unambiguous identification of the labelled chromosome as chromosome 11. Figure 1D shows two chromosomes 11 with a signal on each of the chromatids. Under these ISH conditions, two signals were detected in the two chromosomes 11 and one signal in each of the chromatids of chromosome 11. When the enzymatic detection reaction was allowed to proceed for longer periods, large single, fused signals were observed.

Figure 2 shows the imaging process required for acquisition of numerical data for chromosome 11. An enlarged Giemsa-stained chromosome image was digitized (Fig. 2A) and revealed an obvious trimodal condensation pattern. A green overlay line was interactively drawn on the mid-rib of a chromatid. Similar digitization and psuedo-coloring of an image of the same chromosome after ISH, with a blue overlay line in the same position for reference (Fig. 2B), unequivocally positions the signals on both chromatids.

The signals were superimposed onto the pseudo-colored original image with blue color (Fig. 2C), defining their exact positions on the chromosome before ISH. The signal positions are depicted in the proximal region of the short arm on this integrated image. A pseudo-three dimensional image of the same chromosome with purple signals superimposed (Fig. 2D) also reveals the characteristic trimodal condensation pattern and the signal position on the chromosome.

Figure 3 illustrates the density profiles along the mid-rib of the original chromosomal image, and along the signal image after ISH. The characteristics of chromosome 11 are revealed by the first profile line, while the signal is accurately represented by a smooth profile,

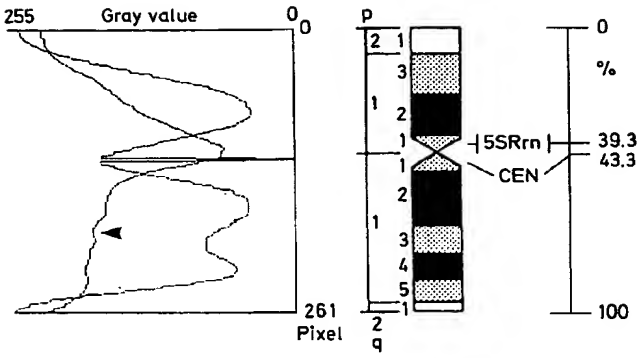


Fig. 3 Density profiles of Giemsa-stained image (left panel, *lower trace*) and signal image indicated by an arrow head. Standard chromosome map of chromosome 11 with the positions of the 5S rRNA gene and the centromere (*right panel*). Vertical lines beside the gene name indicate the standard deviation of the position. The vertical bar on the right indicates the relative chromosome length

indicated by an arrowhead in Fig. 3. A standard map of chromosome 11 and a scale indicating the positions of the 5S rDNA and the distance of the locus from the end of the short arm are also depicted. The locus, designated *5SRrn*, is the first 5S rDNA locus to be physically mapped to position 11p1.1 in rice.

The lengths of the short and long arms and the distance from the center of the signal to the centromere were measured using the overlay lines drawn in Fig. 2B. Table 1 shows the numerical data for 41 measurements of the 44 chromatids with signals on chromosome 11. Three measurements were omitted from the final data, since they were significantly different from the average. The standard deviation of each parameter was low and the average arm ratio of the chromosome was consistent with previous data (Fukui and Iijima 1991). The average signal position was very close to the centromere. In fact, 24.4% of the signals were mapped on the centromere. No signal was observed on the long arm.

Figure 4 shows the nucleotide sequences of DNA fragments that contain the coding and non-coding regions of the 5S rDNA in Nipponbare. The sequences were all homologous to one another, although there were some differences due to deletions or insertions. These sequences are also homologous to the sequence of 5S rDNA in IR-20, an indica rice variety, previously reported by Hariharan et al. (1987), but there were significant sequence differences, particularly in the non-coding regions, due to deletions (or insertions) as well as base substitutions.

Discussion

We have shown here that one 5S rDNA locus of japonica rice is localized in the proximal region of the short arm of chromosome 11 (11p1.1). Chromosome 11 is a metacentric chromosome with an arm ratio of 1.26 ± 0.12 (Fukui and Iijima 1991). Thus the 5S rDNA locus, in close proximity to the centromere, should be located in the central region of the chromosome.

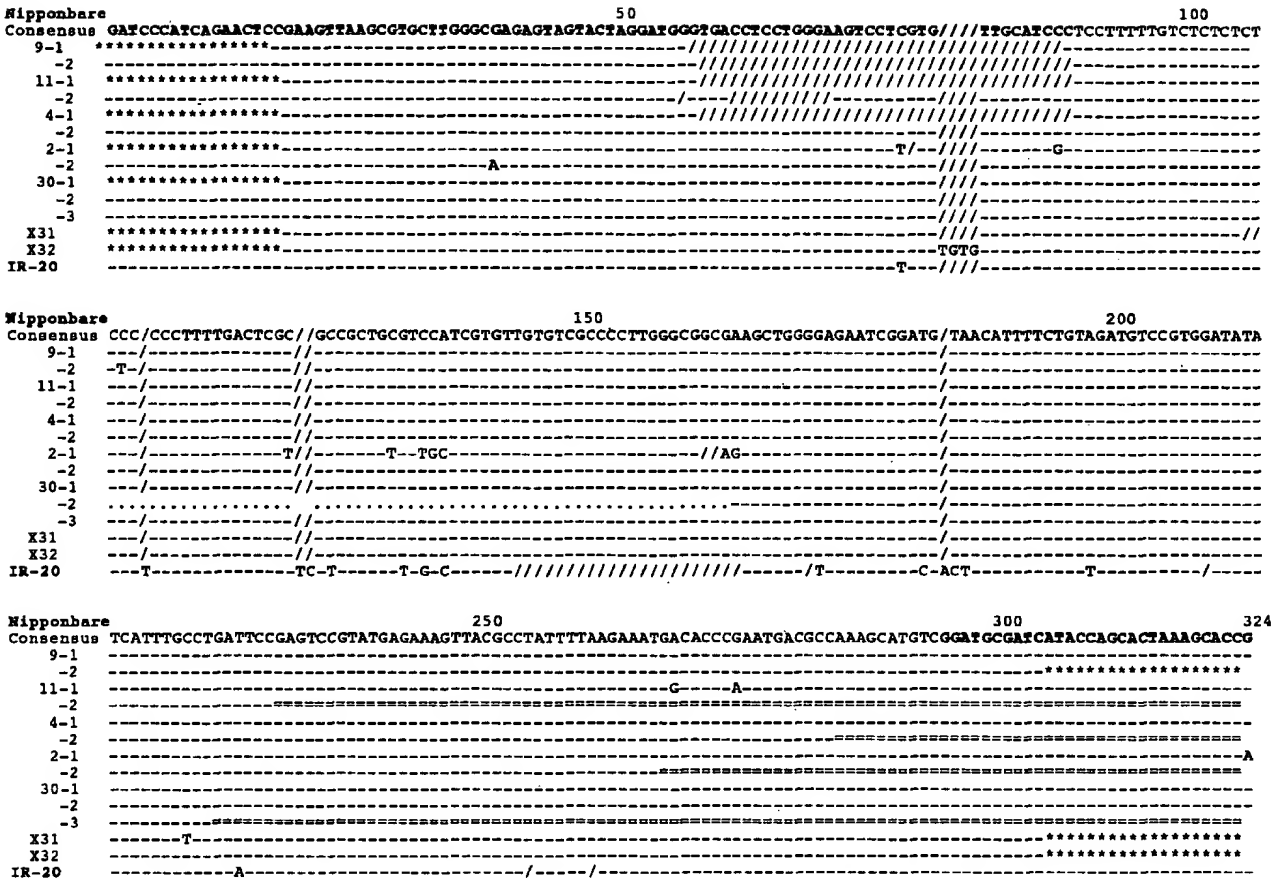
The chromosomal assignment of the 5S rDNA locus obtained by ISH is consistent with that determined by RFLP analysis (McCouch et al. 1988). The 5S rDNA locus was also placed in the center of the linkage map (Tanksley et al. 1992). The close proximity of the 5S rDNA locus to the centromere suggests that cloning of useful genes closely linked to 5S rDNA by chromosome walking from the 5S rDNA may be difficult, since it is known that recombination values around the centromeric region are distorted. Lapitan et al. (1991) showed that, in tomato, the 5S rDNA locus lies on the short arm of chromosome 1, close to the centromere and separate from the rDNA locus. The localization of 5S rDNA at a site distinct from those of rDNA loci and its close proximity to a centromere, observed both in rice and tomato, suggest a certain degree of conservation in overall genome organization in these species.

Chromosome 11 (the 9th longest chromosome in rice) is easily discriminated from the other rice chromosomes under a microscope by its characteristic condensation pattern. Chromosome 11 has been designated as Mitsuyama, or Trimodal (Fukui and Iijima 1991), as there are three heavily condensed regions within the chromosome, one in the proximal region of the short arm and the other two in the long arm, which are observed with an appearance frequency of 96.7% (Fukui and Iijima 1992). This characteristic, unique among the rice chromosomes, paved the way for accurate identification of the chromosome in the complement. The

Table 1 Location of *5SRrn* on rice chromosome 11

Length (μm)			Section length	Number of chromatids observed
Short arm	Long arm	Total		
1.39 ± 0.21 (43.3 ± 3.5)[b]	1.82 ± 0.23 (56.7 ± 3.5)	3.21 ± 0.38 (100)	0.13 ± 0.09[a] (4.00 ± 2.6)	41

[a] Distance of signal in the short arm from the centromere
[b] Figures in parentheses indicate percentages



fact that, in most of the chromosomal spreads, both homologues had signals and label was seldom found on one chromosome only, also confirmed the effectiveness of the cytological and FISH methods currently employed.

Song and Gustafson (1993) have recently reported the results of physical mapping of 5S rDNA in indica rice by ISH. They located one 5S rDNA locus at the distal end of the short arm of chromosome 9, consistent with the data obtained by trisomic dosage analysis (McCouch et al. 1988, cited by Song and Gustafson 1993) and with linkage of 5S rDNA to a tolemeric probe [S. D. Tanksley (unpublished data), cited by Song and Gustafson 1993]. McCouch et al. (1988) localized the 45S rDNA (17S, 5.8S and 25S ribosomal RNA) gene complex at the terminus of the RFLP map of chromosome 9, while the 5S rDNA was mapped to chromosome 11. Wu and Tanksley (1993) have physically mapped one telomere region to the distal end of chromosome 11.

The difference in the position of the 5S rDNA loci between japonica and indica rice can be ascribed to the different subspecies used. A difference in the number of

Fig. 4 Nucleotide sequences of 5S rDNA. The sequences of the fragments with one, two, or three units of 5S rDNA from Nipponbare and a sequence from IR-20 (Hariharan et al. 1987) are shown. *Boldface letters* indicate the nucleotide sequences coding for 5S rRNA. *Slashes* indicate the bases missing. Positions of primers are indicated by *. Nucleotide sequences not determined are indicated by *dots*, and those not included in the cloned fragments by *double lines*. The sequence data reported appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number (D26370)

rDNA loci has already been detected between japonica and indica rice (Fukui et al. 1994b). It is possible that there exist in rice other minor 5S rDNA loci, in addition to the locus detected here, which have fewer gene copies and thus lie below the detection limits of the methods used. In barley, one 5S rDNA locus was first reported by Appels et al. (1980). They, however, predicted the possible existence of other loci that could not be detected, due to the limitations of the method used, based on radioactive ISH. In fact, three additional 5S rDNA loci and four/six 5S rDNA-related sites, depending on the variety, have since been revealed using

improved ISH, by Leitch and Heslop-Harrison (1993) and Fukui et al. (1994a), respectively.

Here, we have presented the nucleotide sequences of the coding and non-coding regions of 5S rDNA in the japonica rice variety Nipponbare and compared them with 5S rDNA in an indica rice variety. There were significant differences between the 5S rDNA sequences in the two rice varieties, particularly in their non-coding regions. It is likely that the sequences of 5S rDNA tandemly repeated at the locus in the japonica rice variety differ from those at the locus in indica rice. The sequence differences detected in the highly repeated 5S rRNA genes especially in non-coding regions would also provide clues to understanding chromosome and genome organization in rice.

References

Appels R, Gerlach WL, Dennis ES, Swift H, Peacock WJ (1980) Molecular and chromosomal organization of DNA sequences coding for the ribosomal RNAs in cereals. Chromosoma 78:293–311

Fukui K (1984) In situ hybridization. In: Uchimiya H, Tanaka K, Sugiura A (eds) Experimental manual for plant genetic engineering. Kodansha Sci, Tokyo, pp 165–168

Fukui K (1985) Identification of plant chromosomes by image analysis method. The Cell (Tokyo) 17:145–149

Fukui K (1986) Standardization of karyotyping plant chromosome by a newly developed chromosome image analyzing system (CHIAS). Theor Appl Genet 72:27–32

Fukui K (1988) Analysis and utility of chromosome information by using the chromosome image analyzing system, CHIAS. Bulletin of the National Institute of Agrobiological Resources 4:153–176

Fukui K (1990) Localization of rRNA genes on rice chromosomes. Rice Biotech Quart 1:18–19

Fukui K, Iijima K (1991) Somatic chromosome map of rice by imaging methods. Theor Appl Genet 81:589–596

Fukui K, Iijima K (1992) Manual on rice chromosomes (2nd edition). Miscellaneous Publications of the National Institute of Agrobiological Resources 4:1–25

Fukui K, Ito K (1989) Computer simulation for microscopic images of barley chromosomes. Bulletin of the National Institute of Agrobiological Resources 5:21–35

Fukui K, Kamisugi Y (1991a) Simulation of microscopic images by a desktop imaging system. In: Doyama M, Suzuki T, Kihara J, Yamamoto R (eds) Computer-aided innovation of new materials. Elsevier Science Publishers, Amsterdam, pp 101–104

Fukui K, Kamisugi Y (1991b) Image analysis of chromosomes by computer 1. Cell Sci 7:590–593

Fukui K, Mukai Y (1988) Condensation pattern as a new image parameter for identification of small chromosomes in plants. Jpn J Genet 63:359–366

Fukui K, Kakeda K, Hashimoto J, Matsuoka S (1987) *In situ* hybridization of ^{125}I labeled rRNA to rice chromosomes. Rice Genet Newslett 4:114–116

Fukui K, Kamisugi Y, Sakai F (1994a) Determination of 5SrRNA loci by direct-cloned biotinylated probes in barley chromosomes. Genome 37:105–111

Fukui K, Ohmido N, Khush GS (1994b) Variability in rDNA loci in genus *Oryza* detected through fluorescent *in situ* hybridization. Theor Appl Genet 87:893–899

Hariharan N, Reddy PS, Padayatty JD (1987) 5S-rRNA genes in rice embryos. Plant Mol Biol 9:443–451

Iijima K, Fukui K (1991) Clarification of the conditions for the image analysis of plant chromosomes. Bulletin of the National Institute of Agrobiological Resources 6:1–58

Iijima K, Kakeda K, Fukui K (1991) Identification and characterization of somatic rice chromosomes by imaging methods. Theor Appl Genet 81:576–605

Islam-Faridi MN, Ishii T, Kumar V, Sitch LA, Brar DS (1990) Chromosomal location of ribosomal RNA genes in rice by *in situ* hybridization. Rice Genet Newslett 7:143–144

Kamisugi Y, Fukui K (1992) Image analysis of chromosomes by computer 7. Cell Sci 8:70–75

Lapitan NLV, Ganal MW, Tanksley SD (1991) Organization of the 5S ribosomal RNA genes in the genome of tomato. Genome 34:509–514

Leitch IJ, Heslop-Harrison JS (1993) Physical mapping of four sites of 5S rDNA sequences and one site of the α-amylase-2 gene in barley (*Hordeum vulgare*). Genome 36:517–523

McCouch SR, Kochert G, Wang ZH, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:20–78

Mochizuki K, Umeda M, Ohtsubo H, Ohtsubo E (1992) Characterization of a plant SINE, p-SINE1, in rice genomes. Jpn J Genet 67:155–166

Mukai Y, Endo TR, Gill BS (1990) Physical mapping of the 5S rRNA multigene family in common wheat. J Hered 82:290–295

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Song YC, Gustafson JP (1993) Physical mapping of the 5S rDNA gene complex in rice (*Oryza sativa*). Genome 36:658–661

Tanksley SD, Fulton TM, McCouch SR (1992) Linkage map of rice (*Oryza sativa*) (2N = 24). In: O'Brien SJ (ed) Genetic maps. Locus maps of complex genomes (6th ed.) Book 6. Cold Spring Harbor Laboratory Press, New York, pp 61–79

Wu KS, Tanksley SD (1993) Genetic and physical mapping of telomeres and macrosatellites of rice. Plant Mol Biol 22:861–872

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Patterns of heterochromatin distribution in plant chromosomes

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Abstract

The C-band distribution patterns of 105 angiosperm species were compared to identify general patterns or preferential sites for heterochromatin. The base-specific fluorochrome reaction of heterochromatin for 58 of these species and the role played by the average chromosome size in band distribution were also considered. The results showed that heterochromatin was preferentially located in similar chromosome regions, regardless of the distance from the centromere. This trend results in generalized bands, with heterochromatin distribution being identical in most chromosomes of a karyotype. Such bands very often displayed the same fluorochrome reaction, suggesting possible repeat transfer between non-homologous sites. Chromosome size may also play a role in heterochromatin location, since proximal bands were much more common in small-sized chromosomes.

INTRODUCTION

Heterochromatin (HC) is one of the chromosomal components that has attracted most attention from cytogeneticists, because of its still unknown function, its apparent lack of genes and the fact that it stains differently to the rest of the chromosome. Especially after the development of HC differentiation techniques in the metaphase chromosomes of a great diversity of organisms, a large number of papers were written which reported its presence, variability, molecular composition, direct and indirect effects on the karyotype, putative function, etc. (see Mizianty, 1984a,b, 1985; John, 1988; Hennig, 1999). One of the most basic, but by no means the least important aspects of HC research, is the study of its distribution in blocks or bands throughout the karyotype. Analysis of band distribution patterns can make it possible to verify whether the bands are preferentially located in certain chromosomal regions, suggesting that the HC has a functional purpose.

HETEROCHROMATIN VARIABILITY

The analysis of HC distribution patterns in the chromosome complement of angiosperms in general is hindered by the particularly high variability of HC added to the variability of other karyotype parameters. The comparison of HC band patterns of different species should take into consideration the following aspects: 1) the HC is not homogeneous, varying qualitatively and quantitatively between species; 2) within a single species, polymorphism for the number and size of bands is frequent; 3) the amount of HC varies regardless the amount of euchromatin or the nuclear DNA content; 4) both HC and euchromatin can suffer large changes in a relatively short time; 5) different staining techniques may reveal different HC fractions; 6) very different species may present simultaneous differences in chromosome number, size and morphology, as well as in the amount, composition and distribution of HC (Greilhuber, 1982; Vosa, 1985; John, 1988; Sumner, 1990).

The functional and evolutionary meaning of HC may not be the same for all species, and a single distribution pattern for all angiosperms may not exist, only tendencies or preferential patterns for different genomes and karyotype architectures.

In spite of these difficulties in establishing general models, some authors draw attention to distribution patterns that seem to occur very frequently. Heitz (1933) was the first to observe that, in each species, the HC seemed to be distributed non-randomly in proximal, interstitial and telomeric regions. In *Vicia faba*, for instance, he observed that HC was preferentially located in interstitial regions, while in *Allium cepa* it distribution was preferentially terminal. After performing chromosome analysis of several species of plants and animals, Heitz (1957) considered that such an equilocal distribution is a universal rule.

Lima de Faria (1976a,b, 1983) considered that heterochromatin blocks observed by "cold starvation" or by C-banding, as well as chromomeres, knobs, secondary constrictions and other chromosomal markers and DNA sequences, would occupy preferential sites within the *chromosome field* (the chromosome region between centromere and telomere where specific DNA sequences and chromosome phenomena occur in a specific order). Greilhuber (1979), however, re-examined the available data on the distribution of "cold starved" HC and found no preferential regions.

Later, Loidl and collaborators (Loidl, 1983; Greilhuber and Loidl, 1983; Schweizer and Loidl, 1987) drew attention to the fact that in many species HC blocks tend to be distributed in the arms of different chromosomes at an equal distance starting from the centromere (see also Bennett, 1982). In such cases, the chromosomes would tend to exhibit *equidistant* bands (e.g., on the telomeres of similar-sized arms and in the corresponding interstitial regions

of the longest arms), while, according to Heitz, the distribution of bands would be preferentially equilocal (e.g., on the telomeres of short and long arms). These tendencies would result in different karyotype patterns, with equilocalization producing a generalized banding pattern in all chromosomes of a complement (regardless of size and centromeric index variation), while equidistance would generate different banding patterns in asymmetrical karyotypes and identical patterns in symmetrical karyotypes.

HETEROCHROMATIN CHARACTERIZATION

One of the difficulties in understanding HC distribution is the fact that bands of a single karyotype may be composed of different types of HC, i.e., unrelated families of repetitive DNA with independent distribution patterns (Flavell, 1982). Therefore, it is important to separately evaluate the distribution of each HC type by cyto-molecular techniques, as has been done for rye (Appels *et al.*, 1978), *Scilla* (Deumling and Greilhuber, 1982), barley (Brandes *et al.*, 1995), *Aegilops* (Badaeva *et al.*, 1996) and a few other organisms. Working with conventional cytological techniques, only the HC associated with the nucleolar organizer region (NOR-HC) can be specifically identified, due to secondary constrictions or by silver nitrate impregnation. More reliable recognition of the NOR-HC has recently been achieved by *in situ* hybridization, mainly with pTa71 probe, which is able to localize rDNA 18S-5.8S-26S sites in every kind of plant (see, e.g., Galasso *et al.*, 1996a; Hizume *et al.*, 1992) and at least in some animals (Pendás *et al.*, 1993). An alternative way of distinguishing some HC types is to stain the chromosomes with fluorochromes that have a preferential affinity for AT- or GC-rich DNA. This staining can differentiate the HC in only a few groups, allowing the easy observation of their distribution in the chromosomes.

HC may be revealed by different cytological techniques, with identical or very similar results (Vosa, 1976b; Lozano *et al.*, 1990; Berg and Greilhuber, 1993). Conventional staining techniques may sometimes show heteropycnotic regions that form the so-called *prophase condensation patterns* (Fukui and Mukai, 1988; Ikeda, 1988; Benko-Iseppon and Morawetz, 1993). Such regions have often been confused with HC patterns which may be very different from each other (see Morawetz, 1981a; Guerra, 1988a). The HC revealed by C-banding seems to correspond to the most general conception of HC, coinciding with the sites of late replicating DNA and tandem organized satellite DNA (Baumann, 1971; Appels *et al.*, 1978; Deumling and Greilhuber, 1982; Cortés and Escalza, 1986; Schubert and Rieger, 1991). However, the use of different C-banding techniques may result in very different banding patterns. In *Citrus*, for example, HC blocks were found mostly at terminal regions (Guerra, 1985, 1993a; Miranda *et al.*, 1997; Pedrosa *et al.*, 2000), although Ito *et al.* (1993), using a different technique, found exclusively proximal HC blocks. In corn, Aguiar-Perecin and Vosa (1985) reported C-bands in terminal and subterminal chromosomal regions, coinciding in number and position with the knobs. However, Molina (1981) found only proximal bands in all the chromosomes, while Chow and Larter (1981) found proximal and terminal bands. On the other hand, Carvalho and Saraiva (1993), using another technique (KHG banding), found numerous interstitial bands. In all these cases the bands were clear and unequivocal, and the differences in these results were certainly due to the procedures used, which revealed different chromatin fractions.

Besides the C-banding technique, staining with base-specific fluorochromes has been recognized as a reliable method of distinguishing some types of HC in plants (Vosa, 1970, 1976b; Schweizer, 1976). The fluorochromes quinacrine (Q), Hoechst 33258 (H) and 4'-6-diamidino-2-phenylindole (DAPI) preferentially stain AT-rich HC (Q+, H+ and DAPI+ bands, respectively) while mitramycin (MM) and chromomycin A3 (CMA) preferentially stain GC-rich (MM+ or CMA+) regions (Schweizer, 1976; Sumner, 1990). These same fluorochromes may also negatively stain AT-poor or GC-poor HC blocks, respectively, whereas double staining with two fluorochromes of different base specificity (e.g., CMA/DAPI, CMA/H or MM/DAPI) may highlight staining differences (Schweizer, 1976, 1981; Kenton, 1991). In addition, non-fluorescent DNA-ligands, like dystamycin A, may also be used to intensify the CMA or DAPI staining (Schweizer, 1983; Fuchs *et al.*, 1998). However, not all AT-rich or GC-rich HC reacts equally to these fluorochromes (see e.g., Schwarzacher and Schweizer, 1982; Kenton, 1991; Bennett *et al.*, 1995) and some C-banded positive regions react neutrally with fluorochromes, i.e., they fluoresce with the same brightness as euchromatin (Morawetz, 1986a,b; Röser, 1994; Galasso *et al.*, 1996b; Cuellar *et al.*, 1996). Other C-bands, fluoresce with the same intensity when stained with fluorochromes with different base-specificities, such as CMA and DAPI (Loidl, 1983; Guerra, 1989; Okada, 1991; Berg and Greilhuber, 1993). All these HC types were considered here as *neutral* bands.

In this paper the C-band distribution patterns of 105 species, belonging to 58 dicotyledons and 32 monocotyledons genera, were compared in order to identify general patterns of HC distribution or any preferential sites in which it may occur. Only the results obtained with the C-banding techniques most used in plants (Marks, 1975; Schwarzacher *et al.*, 1980; Gill *et al.*, 1991) will be considered. These techniques are based on chromosome hydrolysis with 45% acetic acid, or dilute hydrochloric acid, followed by denaturation in barium hydroxide, treatment with 2x SSC and Giemsa staining. Information about the reactivity to base-specific fluorochromes is included for 58 of these species. The main questions focused on are: 1) Is there a preferential distribution of C-bands in the proximal, interstitial or telomeric regions? 2) Are band distribution patterns influenced by the average chromosome size of the karyotypes? 3) Is there any relationship between GC-rich or AT-rich HC bands and their chromosomal location? 4) Is the

occurrence of generalized equilocal bands influenced by their base composition, their chromosomal position, or the average chromosome size?

MATERIAL ANALYZED: CRITERIA FOR DATA SELECTION

To evaluate the variability of HC distribution patterns among angiosperms, data were compiled for 105 species belonging to 90 genera (Table I). Each species name was preceded by the first three letters of its family name to help recognize the taxa, along with diploid number, average chromosome size, band position in the chromosomes, occurrence and position of generalized bands and consulted references.

Species were selected for inclusion in this list simply because they were published or unpublished author's original data or because they were cited in papers available to the author. To avoid overrepresenting the banding patterns of more widely studied plant groups, such as *Triticum*, *Hordeum*, etc., only a single species of each genus was considered. However, in genera with large karyotype variation, either in the amount or the distribution of HC, like *Capsicum* and *Scilla*, two or more representatives were included. In these cases preference was given to the species with extreme HC patterns and to those about which data was available for both C-band and fluorochrome staining. The references used were always those with good photographic documentation which was compatible with the description in the text, the photographic data generally being more highly valued than the text description. Papers with no photographic data were not considered.

The sample was divided into three groups according

Table I - C-band distribution in plant karyotypes with small, medium and large average chromosome size (ACS). Generalized bands (G) may be located at the telomeric (T), interstitial (I) and proximal (P) regions. Family names are indicated by the three first letters (ANA = Anacardiaceae, ANN = Annonaceae, etc). Numbers after species names identify the fluorochromes used: 1, DAPI; 2, quinacrine; 3, Hoechst 33258; 4, CMA. Heterochromatin with main base composition identified but without any indication of fluorochrome used was stained with CMA/DAPI. NOR associated bands are identified by number, position (T, I or P), and AT or GC richness; base composition not defined = ?; number in brackets are NOR sites without C-bands. Estimated values are indicated by ca. (circa).

Family-species	2n	ACS	Heterochromatin distribution				G	References
			T	I	P	NOR		
Small chromosomes (<3.0 µm)								
ANA - *Anacardium occidentale*	40	1.1	4GC	-	-	2T-GC	-	Gitaí and Guerra (1999)
ANN - *Porcelia goyazensis*	18	ca.2.8	4	-	6	2T	-	Morawetz (1984)
ANN - *Guatteria schlechtendaliana*	28	ca.2.0	-	-	28N	2GC	P	Morawetz and Waha (1985)
ARA - *Hedera helix*	48	ca.2.5	-	2GC	48N	2I-GC	P	König *et al.* (1987)
ARE - *Johannesteijsmannia altifrons*	34	ca.2.0	-	-	34AT	2T-GC	P	Röser (1994)
ARI - *Thottea siliquosa*	26	ca.1.8	2	-	26	-	P	Morawetz (1985)
AST - *Artemisia capillaris*	18	2.4	6	-	18	-	P	Mendelak and Schweizer (1986)
AST - *Emilia sonchifolia*	10	2.5	-	-	10N	2T-GC	P	Guerra and Nogueira (1990) Guerra, M. (unpublished data)
AST - *Helianthus annuus*	34	ca.2.8	-	-	34N	6T-GC	P	Cuellar *et al.* (1996)
BIG - *Jacaranda macrantha*	36	ca.2.3	4	-	36	4T	P	Morawetz (1982)
BRA - *Arabidopsis thaliana*[1]	10	ca.1.5	-	-	10AT	4AT(?)	P	Ambros and Schweizer (1976); Maluszynska and Heslop-Harrison (1991)
BRA - *Raphanus sativus*	18	ca.3.0	-	-	18	2T	P	Hirai *et al.* (1995)
BRA - *Sinapsis alba*	24	ca.2.0	4	-	24	-	P	Geber and Schweizer (1988)
CAP - *Lonicera caprifolium*	18	ca.2.0	14	-	18	-	P	Benko-Iseppon and Morawetz (1993)
CUC - *Cucumis sativus*	14	ca.1.9	24	-	8	2I	T	Ramachandran *et al.* (1985)
EUP - *Manihot esculenta*	36	1.8	4GC	2N	-	4T-GC	-	Carvalho and Guerra (1999)
FAB - *Arachis hypogaea*	40	2.13	6	10	40	2I	P	Cai *et al.* (1987); Fernández and Krapovickas (1994)
FAB - *Cicer arietinum*	16	ca.2.8	-	2GC + 4AT	16AT	2I-GC	P	Galasso *et al.* (1996a)
FAB - *Medicago sativa*	16	ca.2.3	12	16N	16N	2I-GC	I+P	Masoud *et al.* (1991); Calderini *et al.* (1996)
FAB - *Phaseolus calcaratus*	22	1.4	-	-	22	2T	P	Zheng *et al.* (1991)
FAB - *Vigna ambacensis*	20	ca.2.8	8GC	-	20N	-	P	Galasso *et al.* (1996b)
FAB - *Vigna sesquipedalis*	22	2.0	6	3	22	2T	P	Zheng *et al.* (1991)
MAG - *Liriodendron tulipifera*	38	ca.1.3	28	-	7	-	T	Morawetz (1981a)
MON - *Peumus boldus*	78	2.9	-	-	78	12T	P	Morawetz (1981b)
ORC - *Orchis coriophora*[3]	36	ca.2.5	20AT	-	-	(12I)	-	D'Emerico *et al.* (1996)
RUT - *Boenninghausenia albiflora*	20	ca.1.8	-	-	20	-	P	Guerra (1985b)
RUT - *Citrus hystrix*	18	ca.2.0	ca.13	-	ca.10	2T	-	Guerra (1985b)
RUT - *Coleonema pulchrum*	34	ca.1.5	-	-	12	2T	-	Guerra (1985b)

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Table 1 - Continued

Family-species	2n	ACS	Heterochromatin distribution				G	References
			T	I	P	NOR		
RUT - *Glycosmis pentaphylla*	54	ca.2.0	-	-	-	6T-GC	-	Guerra *et al.* (in press); Guerra, M. and Santos, K.G.B. (unpublished data)
RUT - *Murraya paniculata*	18	ca.2.3	18GC	-	4N	2P-GC	T	Guerra (1985b); Guerra *et al.* (in press)
SAP - *Cardiospermum grandiflorum*	20	2.4	12AT	-	-	4T-GC	-	Hemmer and Morawetz (1990)
SAP - *Serjania subdentata*	24	2.2	12AT	-	-	6T-GC	-	Hemmer and Morawetz (1990)
SOL - *Solanum tuberosum*	24	ca.2.7	10	12	18	2I	-	Pijnacker and Ferwerda (1984)
VEL - *Vellozia pattens*	16	ca.0.8	-	-	16N	4T-GC	P	Melo *et al.* (1997)
Medium chromosomes (>3.0 <5.0 µm)								
ANN - *Annona muricata*	14	ca.3.0	4N	-	6AT+8N	2P-GC	P	Morawetz (1986a)
ANN - *Rollinia pulchrinervis*	42	4.0	-	-	18N+20AT +4GC	4T-GC	P	Morawetz (1986b)
ARE - *Coccothrinax litoralis*	36	ca.3.9	-	2 AT	36N	2T-GC	P	Röser (1994)
ARE - *Schippia concolor*	36	ca.3.9	-	-	36N	2P-GC	P	Röser (1994)
AST - *Crepis capillaris*	6	4.7	4	8	4	2T	T	Siljak-Yakovlev and Cartier (1979)
AST - *Lactuca sativa*	18	ca.3.2	-	20	18	4T	P	Koopman *et al.* (1993)
CAP - *Viburnum opulus*	18	ca.4.0	14GC	ca.10GC	-	2T-GC	T	Benko-Iseppon and Morawetz (1993)
COM - *Callisia* sp.	24	ca.5.5	-	-	24	-	P	Jones and Kenton (1984)
COS - *Costus pulverulentus*	18	ca.3.5	-	-	18AT	2T-GC	P	Guerra (1988a)
FAB - *Lathyrus aphaca*[2]	14	3.9	12AT	18AT	-	(2I)	-	Rees and Hazarika (1969); Ünal *et al.* (1995)
FAB - *Lathyrus tingitanus*[2]	14	4.5	2AT	2GC +24AT	14GC	(2I)	I+P	Rees and Hazarika (1969); Ünal *et al.* (1995)
FAB - *Sesbania tetraptera*	12	4.6	-	-	12N	4T-GC	P	Forni Martins *et al.* (1994); Forni Martins and Guerra (1999)
FAB - *Vicia johannis*	14	ca.4.1	12	-	-	2I	T	Ramsay (1984)
FAB - *Vicia lutea*	14	ca.4.7	14	18	-	2T	T+I	Ramsay (1984)
GOO - *Scaevola taccada*	16	ca.4.0	-	-	16N	2T-GC	P	Morawetz (1986a)
HYA - *Fortunatia arida*[2]	34	3.6	29AT	3AT	-	-	T	Fernandez and Daviña (1991)
HYA - *Muscari comosum*	18	ca.3.0	-	8AT	18AT	2GC(?)	P	Lozano *et al.* (1990)
IRI - *Eleutherine bulbosa*	12	3.3	1N	4AT	12AT	2P-GC	P	Guerra (1988b)
ORC - *Cephalanthera longifolia*	32	3.4	-	2AT	32AT	2I-GC	P	Schwarzacher and Schweizer (1982)
ORC - *Psygmorchis pusilla*	12	3.8	-	-	12N	-	P	Felix and Guerra (in press)
POA - *Milium effusum*	28	3.3	44	-	10	4I	T	Bennett and Thomas (1991)
POA - *Zea mays*	20	ca.4.5	8	20	-	2I	I	Aguiar-Perecin (1985); Aguiar-Perecin and Vosa (1985)
POA - *Zingeria biebersteiniana*[1,2,3]	4	ca.4.9	8N	10N	4AT	2GC	T+ I+P	Bennett *et al.* (1995)
RUB - *Genipa americana*	22	ca.4.0	ca.24GC	ca.12GC	ca.10GC	2T-GC	T	Guerra (1993b); Pierozzi and Mendaçolli (1997)
RUT - *Dictamnus albus*	36	ca.3.5	72	-	-	-	T	Guerra (1985b)
SOL - *Capsicum annuum*	24	4.1	ca.24GC	2GC	-	4T-GC	T	Moscone *et al.* (1993, 1996); Moscone, E. (personal communication)
SOL - *Capsicum pubescens*	24	3.9	2AT +32GC	2GC +2N	24N	4T-GC	T+P	Moscone *et al.* (1993, 1996); Moscone, E. (personal communication)
Large chromosomes (>5.0 µm)								
ADO - *Adoxa moschatellina*	36	5.8	45	1	-	7T	T	Greilhuber (1979)
ALL - *Allium fistulosum*[2]	16	ca.11.5	30GC	4N	ca.14N	2T-GC	T+P	Kamizyô and Tanaka (1978)
ALL - *Allium subvillosum*	28	ca.5.4	26GC	19GC	28GC	6T-GC	T+P	Jamilena *et al.* (1990)
ALL - *Nothoscordum cf pulchellum*	10	14.3	-	-	-	2T-GC	-	Felix, L.P. and Guerra, M. (unpublished data)
ALL - *Nothoscordum fragrans*	19	ca.16.0	2AT	6GC	-	5T-GC	-	Sato and Yoshioka (1984); Crosa (1996)
ALL - *Tulbaghia pulchella*[2]	12	10.5	11GC	-	-	(2P)	T	Dyer (1963); Vosa (1970)
ALS - *Alstroemeria magnifica*	16	7.4	2	11	-	6I	-	Buitendijk *et al.* (1998)
ALS - *Alstroemeria ligtu*	16	14.9	14	ca.46	16	6P	T+I+P	Buitendijk *et al.* (1998)
AST - *Anacyclus "coronatus"*	18	6.0	ca.20	4	18	6T	T+P	Schweizer and Ehrendorfer (1976)
AST - *Anacyclus depressus*	18	6.1	-	-	18	4T	P	Schweizer and Ehrendorfer (1976)

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Table I - Continued

Family-species	2n	ACS	Heterochromatin distribution				G	References
			T	I	P	NOR		
AST - *Artemisia judaica*	16	6.0	16	-	13	6T	T+P	Mendelak and Schweizer (1986)
AST - *Crepis praemorsa*	8	9.2	13	ca.15	-	-	T	Siljak-Yakovlev and Cartier (1979)
AST - *Crepis vesicaria*	8	6.2	-	-	8	2T	P	Guerra (1982)
AST - *Hypochoeris brasiliensis*	8	7.8	-	-	6	2I+2C	P	Ruas *et al.* (1995)
AST - *Santolina* sp.	36	6.1	ca.66	-	ca.32	4T	T+P	Guerra, M. (unpublished data)
BOR - *Buglossoides purpurocaerulea*[2]	16	ca.5.9	20GC	20GC	-	(4I)	T+I	D'Amato *et al.* (1981)
COM - *Gibasis karwinskyana*[1,4]	10	11.2	18AT	10AT	-	2T-GC	T+I	Kenton (1978); Kenton (1991)
FAB - *Vicia faba*	12	ca.9.5	-	ca.46AT	2AT	2I-GC	I	Greilhuber (1975); Fuchs *et al.* (1998)
FAB - *Vicia melanops*	10	ca.11.6	8AT	ca.20AT	-	3I-GC	T	D'Amato *et al.* (1980)
HYA - *Hyacinthoides italica*	16	11.2	12	ca.24	16	2P	I+P	König and Ebert (1997)
HYA - *Ornithogalum tenuifolium*[2]	6	5.1	-	-	-	2P-GC	-	Vosa (1997)
HYA - *Prospero autumnale*	12	ca.7.7	-	8AT	12AT	2GC	P	Ebert *et al.* (1996)
HYA - *Puschkinia scilloides*	10	5.5	6	-	-	2T+4I	-	Greilhuber and Speta (1976)
HYA - *Scilla mischtschenkoana*	12	10.0	1AT	ca.14 AT	-	4T-GC	I	Greilluber and Speta (1978); Deumling and Greilhuber (1982)
HYA - *S. persica*	8	13.8	-	-	-	2T	-	Greilhuber and Speta (1978)
HYA - *S. siberica*	12	15.0	10GC	5GC	12AT	2P-GC	T+P	Greilhuber and Speta (1978); Deumling and Greilhuber (1982)
LIL - *Bulbine alata*	28	5.1	36	4	28	2T	T+P	Watson (1988)
LIL - *Lilium canadense*	24	ca.16.0	-	50	24	4T	I+P	Smyth *et al.* (1989)
LIL - *Tulipa gesneriana*[2,3,4]	24	ca.11.0	36	ca.36	-	-	T+I	Blakey and Vosa (1982)
ORC - *Cypripedium segawai*	20	ca.13.8	24AT	61AT	20AT	(2P)	T+I+P	Kondo *et al.* (1994); Hoshi *et al.* (1995)
PAE - *Paeonia tenuifolia*	10	19.1	-	-	10N	6T-GC	P	Schwarzacher-Robinson (1986)
POA - *Dasypyrum villosum*	14	ca.6.0	ca.26AT	ca.26AT	ca.14AT	2T-GC	T+I+P	Cremonini *et al.* (1994); Pignone *et al.* (1995)
POA - *Festuca rubra*	42	ca.5.5	ca.70	-	-	-	T	Bailey and Stace (1992)
POA - *Hordeum vulgare*	14	ca.11.7	ca.20	ca.62	-	4I	T+I	Kakeda *et al.* (1991)
POA - *Secale cereale*[3]	14	9.1	28AT	ca.40AT-N	14N	2I-GC	T+I+P	Sarma and Natarajan (1973); Mukai *et al.* (1992)
POA - *Triticum longissimum*	14	ca.9.0	28	92	14	4I	T+I+P	Friebe *et al.* (1993)
RAN - *Aconitum sanyoense*	16	ca.6.0	4GC +14AT	4AT	6AT-2N	2T-GC	T	Okada (1991)
RAN - *Anemone blanda*[1]	16	ca.12.0	-	40AT	-	2T	I	Marks and Schweizer (1974); Hagemann *et al.* (1993)
RAN - *Helleborus foetidus*[2]	32	ca.7.4	-	-	-	6T-GC	-	D'Amato and Bianchi (1989)
RAN - *Nigella damascena*	12	ca.11.0	-	-	12	2T	P	Marks (1975)
SAL - *Azima tetracantha*	22	9.2	ca.28N	ca.6N	22N	2I-GC	T+P	Guerra (1989)
SOL - *Cestrum fasciculatum*	16	ca.6.0	-	ca.7GC +16AT	14N	4T-GC	I+P	Berg and Greilhuber (1993)
SOL - *Cyphomandra luteoalba*	24	6.1	10	46	-	2T	I	Pringle and Murray (1993)
TRI - *Paris tetraphylla*	10	22.7	8	6	10	2T	T+P	Miyamoto and Kurita (1990)

to average chromosome size, based on the fact that the heteropycnotic characteristics of the chromatin of species with small, medium and large chromosomes are frequently different, resulting in different structural types of interphase nuclei (Delay, 1949; Guerra, 1987). Small chromosomes (<3.0 µm) usually have arreticulate or pro-chromosome nuclei, which present early condensed proximal areas in prophase, e.g., heterochromatic blocks. On the other hand, large chromosomes (>5.0 µm) usually have reticulate or eureticulate nuclei, with more or less homogeneous chromosome condensation. Medium-sized chromosomes (>3.0 <5.0 µm) display semi-reticulate nuclei with irregular and variable chromosome condensation (Delay, 1949; Guerra, 1987). This correlation is most evident between DNA amount and nuclear interphase type (Barlow, 1977). However, since no DNA amount estimates were available for many of the species included, nuclear interphase type was correlated with average chromosome size.

The chromosome size was preferentially based on measurements supplied by the referred authors, although in the many cases where the chromosome size was not referred to in the paper, but a scale bar was presented in the photos or idiograms, a length estimation was made with the help of the scale. In these species the calculated chromosome size are more inaccurate, since such photographs show the banding pattern, but not necessarily with a representative chromosome size. In Table I these values are indicated as circa (ca.).

Whenever possible, the chromosome size estimated from conventionally stained cells was adopted, since the

procedure used for C-banding or fluorochrome staining can apparently change the chromosome size. Schwarzacher and Schweizer (1982), for instance, reported an increase in size of up to 50% for chromosomes of *Cephalanthera* cells after C-banding, in comparison with those stained with Feulgen, while Moscone *et al.* (1996) observed that *Capsicum* chromosomes stained with fluorochrome could be up to 1/3 larger than conventionally stained ones. On the other hand, *Cyphomandra* chromosomes treated with C-banding techniques were reduced to a third of the size of those stained with orcein (Pringley and Murray, 1993). In some cases, as in *Arabidopsis thaliana* and *Arachis hypogaea*, chromosome measurements made by other authors with conventional staining were used, since it was considered that any errors resulting from intraspecific polymorphism would be smaller than those arising from the measurement of a single cell. In any case, the chromosome size presented is just an approximation of the real size, used to separate the species into the three groups.

In this paper the position of the bands was classified as *proximal* when located in the proximal region or immediately after the primary constriction, as *telomeric* when located in the terminal regions of the chromosome, and as *interstitial* or *intercalar* when they occupied neither of the chromosome arm extremities. When the band occupied the whole chromosome arm, it was classified as telomeric or proximal depending on the dominant band pattern in the karyotype.

Concerning HC reactivity in relation to base-specific fluorochromes, the bands were classified as AT, GC or N (for neutral), depending on how they reacted with fluorochromes that preferentially stain DNA sequences rich in AT or GC. Species analyzed with the fluorochromes DAPI, quinacrine, Hoechst 33258 or CMA are indicated in Table I by the numbers 1, 2, 3 or 4, respectively, after the species name. The other species with an indication of main base composition but without a number were stained with CMA plus DAPI.

The bands associated with the NOR can involve the secondary constriction and/or the chromatin adjacent to this region, and for this kind of band the description in the papers was more important than photographs, since secondary constrictions have very variable expression and may not be expressed in that cell (Sato *et al.*, 1980).

RESULTS AND DISCUSSION

In spite of the great variability known in HC distribution patterns, drastic and discontinuous changes within a related group of species are not common. In most of the genera in which data were available for at least five species, the number of bands and the HC amount varied but the general pattern was relatively well conserved, as in *Anacyclus* (Schweizer and Ehrendorfer, 1976), *Secale* (Bennett *et al.*, 1977), *Vigna* (Zheng *et al.*, 1991; Galasso *et al.*, 1996b) and *Citrus* (Guerra, 1993a; Miranda *et al.*, 1997). The diversity was much higher in genera with karyotypes exhibiting numerous interstitial bands, like *Anemone* (Marks and Schweizer, 1974), *Scilla* (Greilhuber, 1982), and *Tulipa* (Blakey and Vosa, 1982). Data from very different, non-angiosperm taxa, like some gymnosperm or bryophyte genera, also show similar tendencies (see Tanaka and Hizume, 1980; MacPherson and Filion, 1981; Newton, 1986a,b; Davies *et al.*, 1997).

Considering Table I as a representative sample of angiosperms, the genera and species most frequently analyzed with C-banding techniques are those with large chromosomes, like *Triticum*, *Gibasis*, *Allium*, *Scilla*, etc. Most of these genera are monocotyledons, whereas those with small chromosomes and only a few studied species are mainly dicotyledons. The diploid number of the sampled species is quite representative of angiosperms, although very high numbers are not represented. No relationship between chromosome number and any HC characteristic was found.

Frequency of telomeric, interstitial, and proximal C-bands

Table II presents the frequency of species with telomeric, interstitial or proximal C-bands, in relation to chromosome size. In general, C-bands were slightly more frequent in the proximal region than in other regions. Considering only the karyotypes with HC restricted to a single chromosome region one can observe that it was found in 33 of the 105 species, 23 of which had proximal bands, although these numbers may contain some bias. The frequency of telomeric bands may be overestimated due to the common occurrence of NOR-HC in this region, which is not always identified with this technique and may be wrongly counted as common C-bands. On the other hand, the very reduced frequency of interstitial HC blocks in small chromosomes may be partly due to technical difficulties in localizing these bands on the arms of very small chromosomes.

The distribution of C-bands seems to depend at least partly on chromosome size. Proximal bands, in general, were more common in karyotypes with small chromosomes, decreasing in frequency with increasing chromo-

Table II - Number and percentage of species analyzed in Table I with telomeric, interstitial or proximal bands.

Average chromosome size	Number of species in each size category (%)	Band position		
		Telomeric	Interstitial	Proximal
Small	34 (32.4)	20 (58.8)*	7 (20.6)	28 (82.4)
Medium	27 (25.7)	16 (59.3)	16 (59.3)	19 (70.4)
Large	44 (41.9)	30 (68.2)	30 (68.2)	25 (56.8)
Total	105	66	53	72

*The sum of percentages in each size category is over 100 because some species have bands in two or three positions.

some size (Table II). Furthermore, karyotypes with only proximal bands were found in 11 of the 34 species with small chromosomes but in only 12 of the 71 species with large and medium-sized chromosomes, suggesting that this region develops or conserves C-bands more frequently.

The predominance of proximal bands in karyotypes with small chromosomes is more evident in the distribution of *generalized equilocal bands*, considered here as those bands occurring in the same region in at least 80% of the chromosomes of a karyotype. In general, such bands were less common in species with small chromosomes (Table III). However, among 24 species with small chromosomes and generalized equilocal bands, 21 showed generalization in the proximal region, three in the telomeric region and only one in the interstitial region. Such contrast is far less evident in species with medium or large chromosomes (Table III). Very often generalized proximal bands are largely conserved throughout a genus, as in *Crepis* (Ikeda, 1988) or *Sesbania* (Forni-Martins and Guerra, 1999). The frequencies of generalized bands in Table III are overestimated due to the *a priori* selection of species with the maximum number of bands in those genera with a single representative, although this bias was the same for every chromosome size class.

Generalized proximal HC has also been observed in several species only when using modified C-banding methods, as in onion (Fiskesjo, 1974; Cortés and Escalza, 1986), or after *in situ* hybridization, as in *Vigna unguiculata* (Guerra *et al.*, 1996), or after sequential CMA/DAPI staining and C-banding, as in *Cardiospermum grandiflorum* (Hemmer and Morawetz, 1990). In some other species proximal C-bands have been observed only occasionally. Figure 1 illustrates the occurrence of occasional C-bands in *Santolina* (Asteraceae-Anthemidae). In two diploid spe-

Table III - Number and percentage of species with generalized equilocal bands in different chromosomal positions for chromosomes of different sizes (based on Table I).

Average chromosome size	Number of species		Band position		
	Total	With generalized bands	Telomeric	Interstitial	Proximal
Small	34	24 (70.6)*	03 (12.5)*	01 (4.2)	21 (87.5)
Medium	27	26 (96.3)	11 (42.3)	04 (15.4)	16 (61.5)
Large	44	36 (81.8)	23 (63.8)	16 (44.4)	23 (63.8)
Total	105	86 (81.9)	37 (43.0)	21 (24.4)	59 (65.1)

*The sum of percentages for each size category is over 100 because some species have generalized bands in two or three positions.

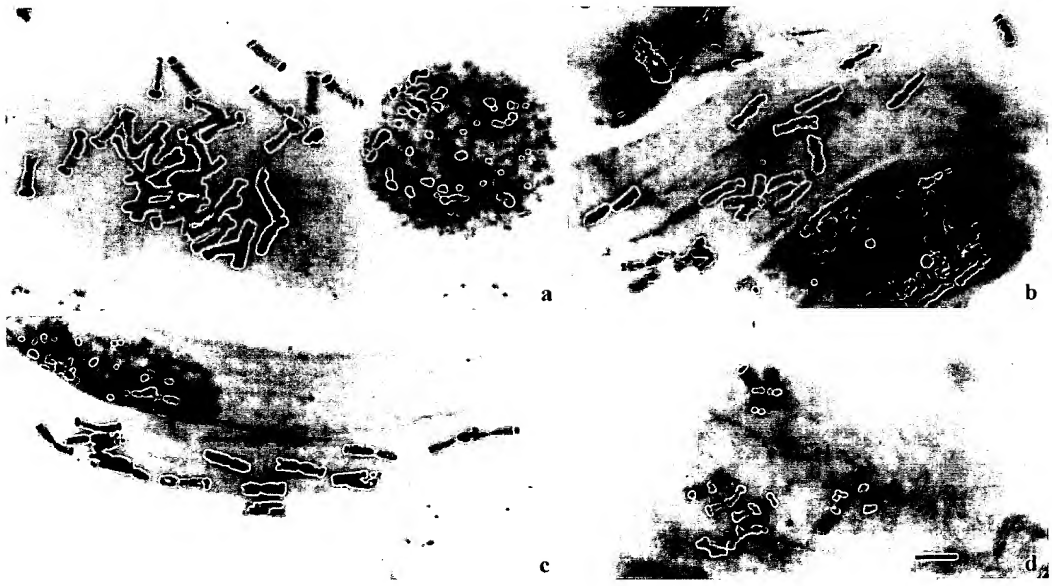

Figure 1 - C-banded metaphase chromosomes of *Santolina* species. **a**, *Santolina* sp. (2n = 36). **b**, *S. rosimarinifolia* (2n = 18). **c**, **d**, *S. chamaecyparissus* (2n = 18). Note proximal bands in most chromosomes of the tetraploid species and at the partial metaphase in **d**. Bar in **d** represents 5 μm.

cies, *S. rosimarinifolia* and *S. chaemacyparissus*, only telomeric bands were found, whereas in a tetraploid, *Santolina* sp., telomeric and proximal bands were always observed (Figure 1a-c). However, in a single slide of *S. rosimarinifolia*, proximal bands were found in all chromosomes (Figure 1d). Such occasional results are certainly not rare, but usually go unreported. These data suggest that the occurrence of any detectable kind of proximal HC is much higher than that observed here, and it may be a common feature of all angiosperm chromosomes (see also Vosa, 1985).

Another interesting aspect is that the occurrence of generalized bands in the interstitial region was not only much lower but almost always accompanied by generalization in another region, since 15 of 20 species with generalized insterstitial bands also presented generalization in the telomeric or proximal region.

NOR associated heterochromatin

Secondary constrictions were not observed or not mentioned in only 14 of the 105 species of Table I. They are usually more difficult to visualize when they are very small or terminally located in the chromosomes (Guerra *et al.*, 1997; Pedrosa *et al.*, 2000). In general, the secondary constrictions were located in the short arm, close to the telomere, as observed by Lima de Faria (1976a), and the NOR-HC was located distally, occupying one or both sides of the constriction.

The NOR-HC is clearly differentiated from other HC types because it contains rDNA genes, although it may also contain repeated sequences not related to the NOR, such as the 120-bp repeat unit of rye (Cuadrado and Jouve, 1994). Analysis of rDNA site distribution by *in situ* hybridization shows that they are always present in the NOR and often in other regions where secondary constrictions have not been located. In *Vigna unguiculata*, for instance, only one or two chromosomes with secondary constrictions were observed, whereas 10 rDNA sites were revealed by FISH (Guerra *et al.*, 1996), eight of them terminally located. NOR-HC often stains differently from other HC types (Sato *et al.*, 1979; Guerra, 1988a) and in extreme cases it does not react positively after C-banding or fluorochrome staining, as in *Tulbaghia pulchella* (Vosa, 1970), *Buglossoides purpurocaerulea* (D'Amato *et al.*, 1981), *Cyprepedium segawai* (Kondo *et al.*, 1994; Hoshi *et al.*, 1995), *Lathyrus aphaca*, *L. tingitanus* (Ünal *et al.*, 1995) and *Orchis coriophora* (D'Emerico *et al.*, 1996).

Bands observed with fluorochromes

The most commonly used fluorochromes were CMA and DAPI, and among the 58 species analyzed with fluorochromes in Table I, 44 were stained with this fluorochrome combination, almost always using the double-staining technique proposed by Schweizer (1976). In some cases, besides CMA and DAPI, other fluorochromes were also used as primary stains (Hoshi *et al.*, 1995; Sato and Yoshioka, 1984) or as counterstains (Deumling and Greilhuber, 1982; Jamilena *et al.*, 1990; Cuellar *et al.*, 1996). Quinacrine was the third most frequently used fluorochrome (10 species), followed by Hoechst 33258.

Bands observed with fluorochromes were also positively stained after C-banding, although not all C-bands were differentiated by fluorochromes. In some species, however, a small number of fluorescent bands were found at sites where C-bands were not detected, as in *Aconitum sanyoense* (Okada, 1991) and *Cestrum fasciculatum* (Berg and Greilhuber, 1993). This fact, together with the apparent lack of NOR associated C-bands in some species, indicates once again that not all heterochromatin is detectable with the most usual C-banding techniques (see, Vosa, 1985).

The relationship between the intensity of the brightness of a fluorochrome stained chromatin and its base composition was originally established for cells not treated with C-banding or any other treatment promoting DNA denaturation-renaturation or preferential extraction of the euchromatin (Casperson *et al.*, 1969; Schweizer, 1976; Sumner, 1990). However, some authors have used fluorochrome staining after the C-banding technique, obtaining bright bands which may not be an indication of AT- or GC-richness (Silva and Guerra, 1998). Bennett *et al.* (1995), for instance, after chromosome staining with quinacrine or DAPI of *Zingeria biebersteiniana* (n = 2), observed fluorescent bands only in the proximal region of one chromosome pair. Using C-banding plus the same fluorochromes, they observed proximal DAPI+ bands in both chromosome pairs and a terminal band in one of them, coinciding with Giemsa stained C-bands. These same DAPI+ bands were also observed after *in situ* hibridization. Vosa (1976a,b) observed that the NOR-HC of some species was positive for Hoechst and quinacrine after C-banding but negative for the same fluorochrome without pretreatment. Such fluorescent bands obtained only after C-banding were considered as neutral in Table I.

The most characteristic HC detected with fluorochromes was the NOR-HC, almost always CMA+ and DAPI-. The reaction intensity of CMA with this HC was variable. Most frequently it was more intense than with other GC-rich bands, but in *Muscari comosum* and *Psygmorchis pusilla* it was faintly stained or neutral. Interestingly, in *Arabidopsis thaliana* the NOR associated HC was apparently positive after staining with DAPI (Maluszynska and Heslop-Harrison, 1991; Ross *et al.*, 1996), although its reaction with CMA is unknown. In this case, staining with CMA or mithramycin is important to verify whether or not a small CMA+ region exists in the secondary constriction itself or very close to it. Positive bands for DAPI or Hoechst, very close to the NOR, have been reported in several other species (Kenton, 1991; Cremonini *et al.*, 1994).

Distribution of fluorochrome bands

Most species have at least one pair of CMA+ bands, since all of them have at least one pair of NORs (Moravetz, 1986a; Röser, 1994; Guerra *et al.*, in press). In some species there are one or two pairs of CMA+ blocks at a very similar chromosome position to that of the NOR, although no secondary constriction is observed associated with them. This occurs in *Hedera helix* (König *et al.*, 1987), *Cicer arietinum* (Galasso *et al.*, 1996a) and *Hypochoeris chillensis* (Cerbah *et al.*, 1995). However, after *in situ* hybridization with rDNA probes, these CMA bands are also often labelled, suggesting that it is the same type of HC (Galasso *et al.*, 1996a; Cerbah *et al.*, 1998).

In Table I, excluding the NOR bands, AT-rich blocks were found in 29 species, GC-rich in 19 species and neutral blocks in 23 species. Note that the total number of AT- or GC-rich sites per karyotype is not under consideration, but rather the number of species exhibiting at least one such site per chromosomal region. AT-rich sites were more frequently found at the interstitial region in species with medium and large chromosomes size (Table IV). Particularly in the case of bimodal karyotypes with long acrocentrics and small metacentrics or acrocentrics, interstitial bands were almost always AT-rich, as in some *Brimeura* species (Vosa, 1979), *Fortunatia arida*, *Muscari comosum*, *Ornithogalum tenuifolium*, *Eleutherine bulbosa* and *Cephalanthera longifolia* (Table I). A noteworthy exception was *Nothoscordum fragrans*, with interstitial bands that were positive for mithramycin and chromomycin A3 and negative for quinacrine (Sato and Yoshioka, 1984). Other karyotypes basically composed of long acrocentrics, like *Anemone blanda* and *Vicia faba* (Table I), were also characterized by interstitial AT-rich bands.

Among GC-rich bands, some were certainly non-identified rDNA sites, suggesting that the proportion of species with AT-rich HC is even higher. Nevertheless, in some taxa, GC-rich bands are very diversified and highly dominant or even exclusive, as in the genus *Capsicum* (Moscone *et al.*, 1996), in the subfamily Aurantioideae (Guerra *et al.*, in press) or in the family Lemnaceae (Geber, 1989).

Equilocal bands almost always stain in the same way with fluorochromes, suggesting that most of these bands are made up of the same or very similar repeats. Analyses in several organisms with *in situ* hybridization of specific satellite DNA fractions have shown that such repeats usually have equilocal distribution (Appels *et al.*, 1978; Badaeva *et al.*, 1996; Brandes *et al.*, 1995). In *Aegilops*, for instance, a specific satellite sequence was observed in the terminal and subterminal regions of all species investigated, as well as in other chromosomal regions (Badaeva *et al.*, 1996). In some species, two or more unrelated satellite DNAs have been observed occupying the same equilocal sites (Brandes *et al.*, 1995), which may explain those sites that stain positively with different base specific fluorochromes.

Table IV - Distribution of C-bands with different base composition in karyotypes of small, medium and large chromosomes (based on Table I).

Band position	Main base composition	Average chromosome size			Total
		small	medium	large	
Telomeric	GC	04	04	06	14
	AT	03	04	08	15
	N	-	03	01	04
Intercalar	GC	02	05	05	12
	AT	01	07	11	19
	N	02	02	03	07
Proximal	GC	-	03	01	04
	AT	03	07	05	15
	N	08	08	06	22
Total	GC	06	12	12	30
	AT	07	18	24	49
	N	10	13	10	33

CONCLUDING REMARKS

The analysis of C-band patterns in a sample of over one hundred angiosperm karyotypes with different chromosome sizes showed that HC is not randomly distributed but rather that it is preferentially located. The NOR-HC is a notorious example, with a very marked distribution at subtelomeric sites. This HC type is very frequently, but not always, positively stained by C-banding techniques and by fluorochromes with high affinity for GC-rich chromatin. The remaining HC is mainly AT-rich and, regardless of its DNA base composition, it has a generalized equilocal distribution. In small chromosomes the bands are conspicuously preferentially located in proximal regions. Such generalized proximal bands may be related to the high frequency of prochromosomal nuclei in species with small chromosomes and low DNA content (Delay, 1949; Barlow, 1977; Guerra, 1987).

The bands seen here showed a tendency to be preferentially distributed in some specific chromosome regions, regardless of distance from the centromere. Thus, generalized telomeric C-bands were found in similar- and dissimilar-sized arms of a single karyotype, as in *Paris tetraphyla* (Miyamoto and Kurita, 1990), *Aconitum sanyoense* (Okada, 1991) and *Fortunatia arida* (Fernández and Daviña, 1991). Likewise, NOR-HC was terminally located in karyotypes with similar-sized arms, as in *Allium subvillosum* (Jamilena *et al.*, 1990), or different-sized arms, as in *Paeonia tenuifolia* (Schwarzacher-Robinson, 1986). Generalized interstitial C-bands can be also equidistant, as in *Anemone blanda* (Marks and Schweizer, 1974) and *Lathyrus tingitanus* (Ünal *et al.*, 1995), or, more frequently, occupy different positions in interstitial regions, as in several species of *Lilium* (Smyth *et al.*, 1989) and *Cyphomandra* (Pringle and Murray, 1993).

One of the important assumptions in the heterochromatin dispersion model proposed by Schweizer and Loidl (1987) is that interstitial bands in long arms are equidistant to the terminal bands of short arms. Such symmetrical distribution was attributed to HC amplification and transposition occurring during mitotic interphase between non-homologous chromosomes showing Rabl orientation. In the present sample, such interstitial bands corresponding to non-homologous terminal HC were sometimes observed, although often along with several non-equidistant interstitial bands, as in *Gibasis karwinskyana* (Kenton, 1978, 1991) and *Secale cereale* (Mukai *et al.*, 1992). Chromosomes of *Allium subvillosum*, for instance, exhibited telomeric bands in all short arms and interstitial bands in all long arms, but, according to the idiogram of Jamilena *et al.* (1990), the interstitial bands were located at distances almost always larger than the longest of the short arms. Actually, an exact equidistancing is not expected. Since different chromosome arms may have different condensation/decondensation patterns (Okada, 1975; Fukui and Mukai, 1988), the distance between one band and the centromere in non-homologous arms may be quite different in metaphase and in interphase.

These data suggest that generalized bands are not just an accident due to chromatin proximity of more or less equidistant non-homologous regions but most probably are due to the functional or structural similarity of these regions. The equilocal or equidistant chromatin "contamination" by the same type of repetitive DNA sequence may be facilitated, but not determined, by Rabl orientation. This assumption is supported by HC distribution in species with holocentric chromosomes, which are free from the influence of Rabl orientation. Karyotypes of *Drosera* (Sheik and Kondo, 1995, 1996) and *Rhynchospora* (Vanzela *et al.*, in press), genera with holocentric chromosomes and belonging to widely different angiosperm families, exhibit HC distribution patterns similar to those with monocentric chromosomes. In both genera, generalized equilocal C-bands were found, which also gave identical fluorochrome reactions. In several species of *Rhynchospora*, Vanzela *et al.* (1998) observed the occurrence of multiple rDNA sites always distributed in the terminal regions. Similarly, satellite DNA sequences with generalized equilocal distribution were observed in the holocentric chromosomes of the peach potato aphid (Spence *et al.*, 1998; Mandrioli *et al.*, 1999). Therefore, HC equidistribution may not depend fundamentally on Rabl orientation and band-centromere distance, but rather on some structural or functional similarity of each chromosomal region that makes them equally receptive to the installation and/or amplification of the same or similar DNA sequence (Fry and Salser, 1977; Flavell, 1982).

REFERENCES

Aguiar-Perecin, M.L.R de (1985). Bandeamento-C e tipos de heterocromatina em milho. In: *Tópicos de Citogenética e Evolução Vegetal de Plantas* (Aguiar-Perecin, M.L.R., Martins, P.S. and Bandel, G., eds.). Sociedade Brasileira de Genética, Ribeirão Preto, pp. 51-67.

Aguiar-Perecin, M.L.R. and Vosa, C.G. (1985). C-banding in maize II. Identification of somatic chromosome. *Heredity 54*: 37-42.

Ambros, P. and Schweizer, D. (1976). The Giemsa C-banded karyotype of *Arabidopsis thaliana* (L). Heynh. *Arabidopsis Inf. Serv. 13*: 167-171.

Appels, R., Driscoll, C. and Peacock, W.J. (1978). Heterochromatin and highly repeated DNA sequences in rye (*Secale cereale*). *Chromosoma 70*: 67-89.

Badaeva, E.D., Friebe, B. and Gill, B.S. (1996). Genome differentiation in *Aegilops*. 1. Distribution of highly repetitive DNA sequences on chromosomes of diploid species. *Genome 29*: 293-306.

Bailey, J.P. and Stace, C.A. (1992). Chromosome banding and pairing behaviour in *Festuca* and *Vulpia* (*Poaceae*, *Pooideae*). *Plant Syst. Evol. 182*: 21-28.

Barlow, P. (1977). Determinants of nuclear chromatin structure in angiosperms. *Ann. Sci. Nat. Bot. Biol. Veg. 18*: 193-206.

Baumann, T.W. (1971). Heterochromatin und DNS-Replikation bei *Scilla sibirica*. *Exp. Cell Res. 64*: 323-330.

Benko-Iseppon, A.M. and Morawetz, W. (1993). Cold-induced chromosome regions and karyosystematics in *Sambucus* and *Viburnum*. *Bot. Acta 106*: 183-191.

Bennett, M.D. (1982). Nucleotypic basis of the spatial ordering of chromosomes in eukaryotes and the implications of the order for genome evolution and phenotypic variation. In: *Genome Evolution* (Dover, G.A. and Flavell, R.B., eds.). Academic Press, London, pp. 239-261.

Bennett, M.D., Gustafson, J.P. and Smith, J.B. (1977). Variation in nuclear DNA in the genus *Secale*. *Chromosoma 61*: 149-176.

Bennett, S.T. and Thomas, S.M. (1991). Karyological analysis and genome size in *Milium* (Gramineae) with special reference to polyploidy and chromosomal evolution. *Genome 34*: 868-878.

Bennett, S.T., Leitch, I.J. and Bennett, M.D. (1995). Chromosome identification and mapping in the grass *Zingeria biebersteiniana* (2n = 4) using fluorochromes. *Chromosome Res. 3*: 101-108.

Berg, C. and Greilhuber, J. (1993). Cold-sensitive chromosome regions and heterochromatin in *Cestrum* (*Solanaceae*): *C. strigillatum*, *C. fasciculatum*, and *C. elegans*. *Plant Syst. Evol. 185*: 133-151.

Blakey, D.H. and Vosa, C.G. (1982). Heterochromatin and chromosome variation in cultivated species of *Tulipa* subg. *Leiostemones* (Liliaceae). *Plant Syst. Evol. 139*: 163-178.

Brandes, A. Röder, M.S. and Ganal, M.W. (1995). Barley telomeres are associated with different types of satellite DNA sequences. *Chromosome Res. 3*: 315-320.

Buitendijk, J.H., Peters, A., Quené, R.-J. and Ramanna, M.S. (1998). Genome size variation and C-band polymorphism in *Alstroemeria aurea*, *A. ligtu* and *A. magnifica (Alstroemeriaceae)*. *Plant Syst. Evol. 212*: 87-106.

Cai, Q., Lu, S. and Chinnappa, C.C. (1987). Analysis of karyotype and Giemsa C-banding patterns in eight species of *Arachis*. *Genome 29*: 187-194.

Calderini, O., Puppilli, F., Cluster, P.D., Mariani, A. and Arcioni, S. (1996). Cytological studies of the nucleolus organizing regions in the *Medicago* complex: *sativa - coerulea - falcata*. *Genome 39*: 914-920.

Carvalho, R. and Guerra, M. (1999). Citogenética de nove espécies do gênero *Manihot* (Euphorbiaceae). *I Simp. Latin-Am. Citogenét. Evol. Veget.* (Resumos): 13.

Carvalho, C.R. and Saraiva, L.S. (1993). A new heterochromatin banding pattern revealed by modified HKG banding technique in maize chromosome. *Heredity 70*: 515-419.

Caspersson, T., Zech, L., Modest, E.J., Foley, G.E., Wagh, U. and Simonsson, E. (1969). DNA-binding fluorochromes for the study of the organization of the metaphase nucleus. *Exp. Cell Res. 58*: 141-152.

Cerbah, M., Coulaud, J., Godelle, B. and Siljak-Yakovlev, S. (1995). Genome size, fluorochrome banding, and karyotype evolution in some *Hypochoeris* species. *Genome 38*: 689-695.

Cerbah, M., Coulaud, J. and Siljak-Yakovlev, S. (1998). rDNA organization and evolutionary relationships in the *Hypochaeris* (Asteraceae). *J. Hered. 89*: 312-318.

Chow, C. and Larter, E.N. (1981). Centromeric banding in maize. *Can. J. Genet. Cytol. 23*: 255-258.

Cortés, F. and Escalza, P. (1986). Analysis of different banding patterns

and late replicating regions in chromosomes of *Allium cepa*, *A. sativum* and *A. nigrum*. *Genetica 71*: 39-46.

Cremonini, R., Colonna, N., Stefani, A., Galasso, I. and **Pignone, D.** (1994). Nuclear DNA content, chromatin organization and chromosome banding in brown and yellow seeds of *Dasypyrum villosum* (L.) Candargy. *Heredity 72*: 365-373.

Crosa, O. (1996). Sistemática e evolução das espécies da seção *Inodorum Guag.* do gênero *Nothoscordum* Kunth (Allieae, Alliaceae). Doctoral thesis, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Cuadrado, A. and **Jouve, N.** (1994). Mapping and organization of highly repeated DNA sequences by means of simultaneous and sequential FISH and C-banding in 6x-triticale. *Chromosome Res. 2*: 331-337.

Cuellar, T., Belhassen, E., Fernández-Calvín, B., Orellana, J. and **Bella, J.L.** (1996). Chromosomal differentiation in *Helianthus annuus* var. *macrocarpus*: heterochromatin characterization and rDNA location. *Heredity 76*: 586-591.

D'Amato, G. and **Bianchi, G.** (1989). Heterochromatin and Ag-Nor staining in some species of *Helleborus* (Ranunculaceae). *Caryologia 42*: 19-26.

D'Amato, G., Bianchi, G., Capineri, R. and **Marchi, P.** (1980). Heterogeneity of heterochromatic segments and karyotype definition in *Vicia melanops* Sibth & SM. *Caryologia 33*: 347-351.

D'Amato, G., Capineri, R. and **Marchi, P.** (1981). Heterochromatin localization in *Buglossoides purpurocaerulea* (L) I.M. Johnston (Boraginaceae): a further case of correspondence between Q and Feulgen bands. *Caryologia 34*: 395-400.

Davies, B.J., O'Brien, I.E.W. and **Murray, B.G.** (1997). Karyotypes, chromosome bands and genome size variation in New Zealand endemic gymnosperms. *Plant Syst. Evol. 208*: 169-185.

Delay, C. (1949). Recherches sur la structure des noyaux quiescents chez les phanérogames. *Ver. Cytol. Cytophysiol. Veg. 10*: 103-228.

D'Emerico, S., Pignone, D. and **Bianco, P.** (1995). Karyomorphological analysis and heterochromatin characteristics disclose phyletic relationship among 2n = 32 and 2n = 36 species of *Orchis* (Orchidaceae). *Plant Syst. Evol. 200*: 111-124.

Deumling, B. and **Greilhuber, J.** (1982). Characterization of heterochromatin in different species of the *Scilla siberica* group (Liliaceae) by *in situ* hybridization of satellite DNAs and fluorochrome banding. *Chromosoma 84*: 535-555.

Dyer, F.A. (1963). Allocyclic segments of chromosomes and the structural heterozygosity that they reveal. *Chromosoma 13*: 545-576.

Ebert, I., Greilhuber, J. and **Speta, F.** (1996). Chromosome banding and genome size differentiation in *Prospero* (*Hyacinthaceae*): diploids. *Plant Syst. Evol. 203*: 143-177.

Felix, L.P. and **Guerra, M.** Chromosome analysis in *Psygmorchis pusilla* (L.) Dodson et Dressler: the smallest chromosome number known in Orchidaceae. *Caryologia* (in press).

Fernández, A. and **Daviña, J.R.** (1991). Heterochromatin and genome size in *Fortunatia* and *Camassia* (*Hyacinthaceae*). *Kew Bull. 46*: 307-316.

Fernández, A. and **Krapovickas, A.** (1994). Cromosomas y evolución en *Arachis*. *Bonplandia 8*: 187-220.

Fiskesjo, G. (1974). Two types of constitutive heterochromatin made visible in *Allium* by a rapid C-banding method. *Hereditas 78*: 153-156.

Flavell, R. (1982). Sequence amplification, deletion and rearrangement: major sources of variation during species divergence. In: *Genome Evolution* (Dover, G.A. and Flavell, R.B., eds.). Academic Press, London, pp. 301-323.

Forni-Martins, E.R. and **Guerra, M.** (1999) Longitudinal differentiation in chromosomes of some *Sesbania* Scop. species (Fabaceae). *Caryologia 52*: 97-103.

Forni-Martins, E.R., Franchi-Tanibata, N. and **Cardelli de Lucena, M.A.** (1994). Karyotypes of species of *Sesbania* Scop. (*Fabaceae*). *Cytologia 59*: 479-482.

Friebe, B., Tuleen, N., Jiang, J. and **Gill, B.S.** (1993). Standard karyotype of *Triticum longissimum* and its cytogenetic relationship with *T. aestivum*. *Genome 36*: 731-742.

Fry, K. and **Salser, W.** (1977). Nucleotide sequence of HS-α satellite DNA from kangaroo rat *Dipodomys ordii* and characterization of similar sequences in other rodents. *Cell 12*: 1069-1084.

Fuchs, J., Strehl, S., Brandes, A., Schweizer, D. and **Schubert, I.** (1998). Molecular-cytogenetic characterization of the *Vicia faba* genome - heterochromatin differentiation, replication patterns and sequence localization. *Chromosome Res. 6*: 219-230.

Fukui, K. and **Mukai, Y.** (1988). Condensation pattern as a new image parameter for identification of small chromosomes in plants. *Jpn. J. Genet. 63*: 359-366.

Galasso, I., Frediani, M., Cremonini, R. and **Pignone, D.** (1996a). Chromatin characterization by banding techniques, *in situ* hybridization, and nuclear DNA content in *Cicer* L. (Leguminosae). *Genome 39*: 258-265.

Galasso, I., Saponetti, L.S. and **Pignone, D.** (1996b). Cytotaxonomic studies in *Vigna*. III. Chromosomal distribution and reacting properties of the heterochromatin in five wild species of the section *Vigna*. *Caryologia 49*: 311-319.

Geber, G. (1989). *Zur Karyosystematik der Lemnaceae*. Dissertation zur Erlangung des Doktorgrades. Universität Wien, Wien.

Geber, G. and **Schweizer, D.** (1988). Cytochemical heterochromatin differentiation in *Sinapsis alba* (*Cruciferae*) using a simple air-drying technique for producing chromosome spreads. *Plant Syst. Evol. 158*: 97-106.

Gill, B.S., Friebe, B. and **Endo, T.R.** (1991). Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). *Genome 34*: 830-839.

Gitaí, J. and **Guerra, M.** (1999). Análise citogenética de três representantes do gênero *Anarcadium* (Anacardiaceae). *I Simp. Latin-Am. Citogenét. Evol. Veget. (Resumos)*: 25.

Greilhuber, J. (1975). Heterogeneity of heterochromatin in plants: comparison of Hy- and C-bands in *Vicia faba*. *Plant Syst. Evol. 124*: 139-156.

Greilhuber, J. (1979). C-band distribution, DNA content and base composition in *Adoxa moschatellina* (*Adoxaceae*), a plant with cold-sensitive chromosome segments. *Plant Syst. Evol. 131*: 243-259.

Greilhuber, J. (1982). Trends in der Chromosomenevolution von *Scilla* (Liliaceae). *Stapfia 10*: 11-51.

Greilhuber, J. and **Loidl, J.** (1983). On regularities of C-banding patterns, and their possible cause. *Kew Chromosome Conference II*: 344.

Greilhuber, J. and **Speta, L.** (1976). C-banded karyotypes in the *Scilla hohenackeri* group, *S. persica*, and *Puschkinia* (*Liliaceae*). *Plant Syst. Evol. 126*: 149-188.

Greilhuber, J. and **Speta, F.** (1978). Quantitative analyses of C-banded karyotypes, and systematics in the cultivated species of the *Scilla siberica* group (*Liliaceae*). *Plant Syst. Evol. 129*: 63-109.

Guerra, M. (1982). Padrão de bandas C em *Crepis vesicaria* L. *Bol. Soc. Broteriana 55*: 167-174.

Guerra, M. (1985). Cytogenetics of *Rutaceae* III. Heterochromatin patterns. *Caryologia 38*: 335-346.

Guerra, M. (1987). Cytogenetics of *Rutaceae* IV. Structure and systematic significance of interphase nuclei. *Cytologia 52*: 213-222.

Guerra, M. (1988a). Characterization of different types of condensed chromatin in *Costus* (*Zingiberaceae*). *Plant Syst. Evol. 158*: 107-115.

Guerra, M. (1988b). Mitotic and meiotic analysis of a pericentric inversion associated with a tandem duplication in *Eleutherine bulbosa*. *Chromosoma 97*: 80-87.

Guerra, M. (1989). The chromosomes of *Azima tetracantha* (Salvadoraceae). *Plant Syst. Evol. 168*: 83-86.

Guerra, M. (1993a). Cytogenetics of Rutaceae. V. High chromosomal variability in *Citrus* species revealed by CMA/DAPI staining. *Heredity 71*: 234-241.

Guerra, M. (1993b). High amount of heterochromatin in a tropical tree species: *Genipa americana* L. (Rubiaceae). *Cytologia 58*: 427-432.

Guerra, M. and **Nogueira, M.T.M.** (1990). The cytotaxonomy of *Emilia* spp. (*Asteraceae: Senecioneae*) occurring in Brazil. *Plant Syst. Evol. 170*: 229-236.

Guerra, M., Kenton, A. and **Bennett, M.D.** (1996). rDNA sites in mitotic and polytene chromosomes of *Vigna unguiculata* (L.) Walp. and *Phaseolus coccineus* L. revealed by *in situ* hybridization. *Ann. Bot. 78*: 157-161.

Guerra, M., Pedrosa, A., Barros e Silva, A.E., Cornélio, M.T.M., Santos, K. and **Soares Filho, W. dos S.** (1997). Chromosome number and secondary constriction variation in 51 accessions of a *Citrus* germplasm bank. *Braz. J. Genet. 20*: 489-496.

Guerra, M., Santos, K.G.B., Barros e Silva, A.E. and **Ehrendorfer, F.** Cytogenetics of Rutaceae VI: Heterochromatin banding patterns in *Auran-*

tioideae - a case of parallel chromosomal evolution. *Am. J. Bot.* (in press).

Heitz, E. (1933). Die Herkunft der Chromocentren. *Planta 18*: 571-636.

Heitz, E. (1957). Die Chromosomenstruktur im Kern während der Kernteilung und der Entwicklung des Organismus. In: *Conference on Chromosomes*. W.E.J. Tjeenk Willink Verlagsgesellschaft, Zwolle, pp. 1-22.

Hemmer, W. and **Morawetz, W.** (1990). Karyological differentiation in *Sapindaceae* with special reference to *Serjania* and *Cardiospermum*. *Bot. Acta 103*: 372-383.

Hennig, W. (1999). Heterochromatin. *Chromosoma 108*: 1-9.

Hirai, K., Irifune, K., Tanaka, R. and **Morikawa, H.** (1995). Molecular and cytological characterization of a highly repeated DNA sequence in *Raphanus sativus*. *Genome 38*: 1237-1243.

Hizume, M., Ishida, F. and **Murata, M.** (1992). Multiple locations of the rRNA genes in chromosomes of pines, *Pinus densiflora* and *P. thunbergii*. *Jpn. J. Genet. 67*: 389-396.

Hoshi, Y., Kondo, K., Tanaka, R. and **Karasawa, K.** (1995). A comparison of C-banding pattern in nine species of *Cypripedium* (Orchidaceae). *Kromosomo 77*: 2636-2645.

Ikeda, H. (1988). Karyomorphological studies on the genus *Crepis* with special reference to C-banding pattern. *J. Sci. Hiroshima Univ.* (Ser. C) *22*: 65-117.

Ito, Y., Omura, M. and **Nesume, H.** (1993). Improvement of chromosome observation methods for *Citrus*. In: *Techniques on Gene Diagnosis and Breeding* (Hayashi, T., Omura, M. and Scott, N.S., eds.). *FTRS*, Tsukuba, pp. 31-38.

Jamilena, M., Rejón, C.R. and **Rejón, M.R.** (1990). Variation in the heterochromatin and nucleolar organizing regions of *Allium subvillosum* L. (Liliaceae). *Genome 33*: 779-784.

John B. (1988). The biology of heterochromatin. In: *Heterochromatin Molecular and Structural Aspects* (Verma, R.S., ed.). Cambridge University Press, Cambridge, pp. 1-47.

Jones, K. and **Kenton, A.** (1984). Mechanisms of chromosome change in the evolution of the tribe Tradescantieae (Commelinaceae). In: *Chromosomes in Evolution of Eukaryotic Groups* (Sharma, A.K. and Sharma, A., eds). CRC Press, Boca Raton, pp. 143-168.

Kakeda, K., Fukui, K. and **Yamagata, H.** (1991). Heterochromatic differentiation in barley chromosome revealed by C- and N-banding techniques. *Theor. Appl. Genet. 81*: 144-150.

Kamizyô, A. and **Tanaka, N.** (1978). Studies on the generative nuclear division I. Comparative analysis of somatic chromosomes in *Allium fistulosum* by means of C- and Q-banding patterns. *Cytologia 43*: 679-688.

Kenton, A. (1978). Giemsa C-banding in *Gibasis* (Commelinaceae). *Chromosoma 65*: 309-324.

Kenton, A. (1991). Heterochromatin accumulation, disposition and diversity in *Gibasis karwinskyana* (Commelinaceae). *Chromosoma 100*: 467-478.

Kondo, K., Hoshi, Y. and **Tanaka, R.** (1994). Somatic chromosome differentiation in *Cypripedium segawai* Masamune and *C. japonicum* Thunberg. *Cytologia 59*: 115-120.

König, C. and **Ebert, I.** (1997). Computer-aided quantitative analysis of banded karyotypes, exemplified in C-banded *Hyacinthoides italica* s. l. (Hyacinthaceae). *Caryologia 48*: 47-63.

König, C., Ebert, I. and **Greilhuber, J.** (1987). A DNA cytophotometric and chromosome banding study in *Hedera helix* (Araliaceae), with reference to differential DNA replication associated with juvenile-adult phase change. *Genome 29*: 498-503.

Koopman, W.J.M., De Jong, J.H. and **De Vries, I.M.** (1993). Chromosome banding patterns in lettuce species (*Lactuca* sect. *Lactuca*, *Compositae*). *Plant Syst. Evol. 185*: 249-257.

Lima-de-Faria, A. (1976a). The chromosome field I. Prediction of the location of ribosomal cistrons. *Hereditas 83*: 1-22.

Lima-de-Faria, A. (1976b). The chromosome field II. The location of "knobs" in relation to telomeres. *Hereditas 83*: 23-34.

Lima-de-Faria, A. (1983). *Molecular Evolution and Organization of the Chromosome*. Elsevier, Amsterdam.

Loidl, J. (1983). Some features of heterochromatin in wild *Allium* species. *Plant Syst. Evol. 143*: 117-131.

Lozano, R., Jamilena, M., Rejón, C.R. and **Rejón, M.R.** (1990). Characterization of the chromatin of some liliaceous species after digestion with restriction endonuclease and sequential Giemsa, fluorochrome and silver staining. *Heredity 64*: 185-195.

MacPherson, P. and **Filion, W.G.** (1981). Karyotype analysis and the distribution of constitutive heterochromatin in five species of *Pinus*. *J. Hered. 72*: 193-198.

Maluszynska, J. and **Heslop-Harrison, J.S.** (1991). Localization of tandemly repetead DNA sequences in *Arabidopsis thaliana*. *Plant J. 2*: 159-166.

Mandrioli, M., Bizzaro, D., Manicardi, G.C., Gionghi, D., Bassoli, L. and **Bianchi, U.** (1999). Cytogenetic and molecular characterization of a highly repeated DNA sequence in the peach potato aphid *Myzus persicae*. *Chromosoma 108*: 436-442.

Marks, G.E. (1975). The Giemsa-staining centromeres of *Nigella damascena*. *J. Cell Sci. 18*: 19-25.

Marks, G. E. and **Schweizer, D.** (1974). Giemsa banding: karyotype differences in some species of *Anemone* and in *Hepatica nobilis*. *Chromosoma 44*: 405-416.

Masoud, S.A., Gill, B.S. and **Jonhson, L.B.** (1991). C-banding of alfalfa chromosomes: standard karyotype and analysis of a somaclonal variant. *J. Hered. 82*: 335-338.

Melo, N.F., Guerra, M., Benko-Iseppon, A.M. and **Menezes, N.L.** (1997). Cytogenetics and cytotaxonomy of *Velloziaceae*. *Plant Syst Evol. 204*: 257-273.

Mendelak, M. and **Schweizer, D.** (1986). Giemsa C-banded karyotypes of some diploid *Artemisia* species. *Plant Syst. Evol. 151*: 195-210.

Meritt, J.F. (1974). The distribution of heterochromatin in the genus *Nicotiana* (Solanaceae). *Am. J. Bot. 61*: 982-994.

Miranda, M., Ikeda, F., Endo, T., Moriguchi, T. and **Omura, M.** (1997). Comparative analysis on the distribution of heterochromatin in *Citrus*, *Poncirus* and *Fortunella* chromosomes. *Chromosome Res. 5*: 86-92.

Miyamoto, J. and **Kurita, S.** (1990). C-band polymorphism in the karyotype of *Paris tetraphylla* A. Gray (Liliaceae). *Cytologia 55*: 301-313.

Mizianty, M. (1984a). Banding patterns in plant chromosomes. II. Bibliography (1970-1980) - *Anthophyta. Acta Soc. Bot. Pol. 53* (Suppl. 1): 485-487.

Mizianty, M. (1984b). Banding patterns in plant chromosomes. II. Bibliography (1970-1980) - *Anthophyta. Acta Soc. Bot. Pol. 53*: 111-136.

Mizianty, M. (1985). Banding patterns in plant chromosomes. II. Bibliography (1970-1980) - *Anthophyta. Acta Soc. Bot. Poloniae 54* (Suppl. 2): 193-194.

Molina, M.D.C. (1981). Caracterizacíon de los cromosomas de *Zea mays* con bandas C con Giemsa. *Mendeliana 5*: 3-8.

Morawetz, W. (1981a). C-banding in *Liriodendron tulipifera* (*Magnoliaceae*): some karyological and systematic implications. *Plant Syst. Evol. 138*: 209-216.

Morawetz, W. (1981b). Karyologie und ökologisch-morphologische Differenzierung von *Peumus boldus (Monimiaceae, Laurales)*. *Plant Syst. Evol. 150*: 119-141.

Morawetz, W. (1982). Morphologisch-ökologische Differenzierung, Biologie, Systematik und Evolution der neotropischen Gattung *Jacaranda* (Bignoniaceae). *Österr. Akad. Wiss. Denkschriften 123*: 1-184.

Morawetz, W. (1984). How stable are genomes of tropical woody plants? Heterozygosity in C-banded karyotypes of *Porcelia* as compared with *Annona* (*Annonaceae*) and *Drimys (Winteraceae)*. *Plant Syst. Evol. 145*: 29-39.

Morawetz, W. (1986a). Remarks on karyological differentiation patterns in tropical woody plants. *Plant Syst. Evol. 152*: 49-100.

Morawetz, W. (1986b). Systematics and karyoevolution in *Magnoliidae*: *Tetrameranthus* as compared with other *Annonaceae* genera of the same chromosome number. *Plant Syst. Evol. 154*: 147-173.

Morawetz, W. and **Waha, M.** (1985). A new pollen type, C-banded and fluorochrome counterstained chromosomes, and evolution in *Guatteria* and related genera (*Annonaceae*). *Plant Syst. Evol. 150*: 119-141.

Moscone, E.A., Lambrou, M., Hunziker, A.T. and **Ehrendorfer, F.** (1993). Giemsa C-banded karyotypes in *Capsicum* (*Solanaceae*). *Plant Syst. Evol. 186*: 213-229.

Moscone, E.A., Lambrou, M. and **Ehrendorfer, F.** (1996). Fluorescent chromosome banding in cultivated species of *Capsicum* (*Solanaceae*). *Plant Syst. Evol. 202*: 37-63.

Mukai, Y., Friebe, B. and **Gill, B.S.** (1992). Comparison of C-banding pat-

terns and *in situ* hybridization sites using highly repetitive and total genomic rye DNA probes of "Imperial" rye chromosomes added to "Chinese Spring" wheat. *Jpn. J. Genet. 67*: 71-83.

Newton, M.E. (1986a). Bryophyte phylogeny in terms of chromosome cytology. *J. Bryol. 14*: 43-57.

Newton, M.E. (1986b). *Pellia borealis* Lorbeer: its cytological status and discovery in Britain. *J. Bryol. 14*: 215-230.

Okada, H. (1975). Karyomorphological studies of wood Polycarpicae. *J. Sci. Hiroshima Univ.* (Ser. B, Div. 2) *15*: 115-200.

Okada, H. (1991). Correspondence of Giemsa C-band with DAPI/CMA fluorochrome staining pattern in *Aconitum sanyoense* (Ranunculaceae). *Cytologia 56*: 135-141.

Pedrosa, A., Schweizer, D. and **Guerra, M.** (2000). Cytological heterozygosity and the hybrid origin of sweet orange (*Citrus sinesis* (L) Osbeck). *Theor. Appl. Genet. 100*: 361-367.

Pendás, A.M., Morán, P. and **Garcia-Vázquez, E.** (1993). Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. *Cytogenet. Cell Genet. 63*: 128-130.

Pierozzi, N.I. and **Mendaçolli, S.L.J.** (1997). Karyotype and C-band analysis in two species of *Genipa* L. (Rubiaceae, Gardenieae Tribe). *Cytologia 62*: 81-90.

Pignone, D., Galasso, I., Rossino, R. and **Mezzanotte, R.** (1995). Characterization of *Dasypyrum villosum* (L) Candargy chromosomal chromatin by means of *in situ* restriction endonucleases, fluorochromes, silver staining and C-banding. *Chromosome Res. 3*: 109-114.

Pijnacker, L.P. and **Ferwerda, M.A.** (1984). Giemsa C-banding of potato chromosomes. *Can. J. Genet. Cytol. 26*: 415-419.

Pringle, G.J. and **Murray, B.G.** (1993). Karyotypes and C-banding patterns in species of *Cyphomandra* Mart. ex Sendtner (Solanaceae). *Bot. J. Linn. Soc. 111*: 331-342.

Ramachandran, C., Brandenburg, W.A. and **den Nijs, A.P.M.** (1985). Infraspecific variation in C-banded karyotype and chiasma frequency in *Cucumis sativus* (Cucurbitaceae). *Plant Syst. Evol. 151*: 31-41.

Ramsay, G. (1984). C-banding in *Vicia* species. In: *Systems for Cytogenetic Analysis in Vicia faba* L. (Chapman, G.P. and Tarawali, S.A., eds.). Nijhoff/Junk Publishers, Dordrecht, pp. 28-39.

Rees, H. and **Hazarika, M.H.** (1969). Chromosome evolution in *Lathyrus*. *Chromosomes Today 2*: 158-165.

Röser, M. (1994). Pathways of karyological differentiation in palms *(Arecaceae). Plant Syst. Evol. 189*: 83-122.

Ross, K.J., Fransz, P. and **Jone, G.H.** (1996). A light microscopic atlas of meiosis in *Arabidopsis thaliana*. *Chromosome Res. 4*: 507-516.

Ruas, C.F., Ruas, P.M., Matzenbacher, N.I., Ross, G., Bernini, C. and **Vanzela, A.L.L.** (1995). Cytogenetic studies of some *Hypochoeris* species (Compositae) from Brazil. *Am. J. Bot. 82*: 369-375.

Sarma, N.P. and **Natarajan, A.T.** (1973). Identification of heterochromatic regions in the chromosomes of rye. *Hereditas 74*: 233-238.

Sato, S. and **Yoshioka, T.** (1984). Heterogeneity of heterochromatin segments in *Nothoscordum fragrans* chromosomes. *Caryologia 37*: 197-205.

Sato, S., Kuroki, Y. and **Ohta, S.** (1979). Two types of color-differentiated C-banding positive segments in chromosomes of *Nothoscordum fragrans*, Liliaceae. *Cytologia 44*: 715-725.

Sato, S., Ohta, S. and **Kuroki, Y.** (1980). Heteromorphic appearance of acrocentric nucleolus organizer regions in *Nothoscordum fragrans*. *Cytologia 45*: 87-96.

Schubert, I. and **Rieger, R.** (1991). Characterization of plant chromosomes by means of banding techniques, differential incorporation of base analogues and *in situ* hybridization. *The Eukaryotic Chromosome - Structural and Functional Aspects* (Obe, G. and Sobti, A., eds.). Springer Verlag and Narose Publishing House, New Delhi, pp. 31-46.

Schwarzacher, T. and **Schweizer, D.** (1982). Karyotype analysis and heterochromatin differentiation with Giemsa C-banding and fluorescent counterstaining in *Cephalanthera* (Orchidaceae). *Plant Syst. Evol. 141*: 91-113.

Schwarzacher, T., Ambros, P. and **Schweizer, D.** (1980). Application of Giemsa banding to orchid karyotype analysis. *Plant Syst. Evol. 134*: 293-297.

Schwarzacher-Robinson, T. (1986). Meiosis, SC-formation, and karyotype structure in diploid *Paeonia tenuifolia* and tetraploid *P. officinalis*. *Plant Syst. Evol. 154*: 259-274.

Schweizer, D. (1976). Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma 58*: 307-324.

Schweizer, D. (1981). Counterstain-enhanced chromosome banding. *Hum. Genet. 57*: 1-14.

Schweizer, D. (1983). Distamycin-DAPI bands: properties and occurrence in species. *Kew Chromosome Conference II*: 43-51.

Schweizer, D. and **Ehrendorfer, F.W.** (1976). Giemsa banded karyotypes, systematics, and evolution in *Anacyclus (Asteraceae-Anthemideae). Plant Syst. Evol. 126*: 107-148.

Schweizer, D. and **Loidl, J.** (1987). A model for heterochromatin dispersion and the evolution of C-band patterns. *Chromosomes Today 9*: 61-74.

Sheik, A.S. and **Kondo, K.** (1995). Differential staining with orcein, Giemsa, CMA and DAPI for comparative chromosome study of 12 species of Australian *Drosera* (Droseraceae). *Austr. J. Bot. 82*: 1278-1286.

Sheik, A.S. and **Kondo, K.** (1996). Comparative C-banding and fluorescent-banding analysis of seven species of *Drosera* (Droseraceae). *Cytologia 6*: 383-394.

Siljak-Yakovlev, S. and **Cartier, D.** (1979). Utilisation de la colaration différentielle au Giemsa dans l'analyse des caryotypes de quatre *Crepis*. *Rev. Cytol. Biol. Végét. - Bot. 2*: 13-20.

Silva, A.E.B. and **Guerra, M.** (1998). Influência do processo de bandeamento C sobre a coloração com os fluorocromos CMA e DAPI em *Emilia sonchifolia* e *Zephyrantes* sp. *Genet. Mol. Biol.* 21 (Suppl.): 161.

Smyth, D.R., Kongsuwan, K. and **Wisudharomn, S.** (1989). A survey of C-band patterns in chromosomes of *Lilium (Liliaceae). Plant Syst. Evol. 163*: 53-69.

Spence, J.M., Blackman, R.L., Testa, J.M. and **Ready, P.D.** (1998). A 169 bp tandem repeat DNA marker for subtelomeric heterochromatin and chromosomal re-arrangement in aphids of the *Myzus persicae* group. *Chromosome Res. 6*: 167-175.

Sumner, A.T. (1990). *Chromosome Banding*. Unwin Hyman, London.

Tanaka, R. and **Hizume, M.** (1980). C-banding treatment for the chromosomes of some gymnosperms. *Bot. Mag. 93*: 167-170.

Ünal, F., Wallace, A.J. and **Callow, R.S.** (1995). Diverse heterochromatin in *Lathyrus*. *Caryologia 48*: 47-63.

Vanzela, A.L.L., Cuadrado, A., Jouve, N., Luceño, M. and **Guerra, M.** (1998). Multiple locations of the rDNA sites in holocentric chromosomes of *Rhynchospora* (*Cyperaceae*). *Chromosome Res. 6*: 345-349.

Vanzela, A.L.L., Guerra, M. and **Luceño, M.** Heterochromatin differentiation in holocentric chromosomes of *Rhynchospora* (Cyperaceae). *Genet. Mol. Biol.* (in press).

Vosa, C.G. (1970). Heterochromatin recognition with fluorochromes. *Chromosoma 30*: 366-372.

Vosa, C.G. (1976b). Heterochromatin classification in *Vicia faba* and *Scilla sibirica*. *Chromosomes Today 5*: 185-192.

Vosa, C.G. (1979). Heterochromatic banding patterns in the chromosomes of *Brimeura* (*Liliaceae*). *Plant Syst. Evol. 132*: 141-148.

Vosa, C.G. (1985). Chromosome banding in plants. In: *Chromosome and Cell Genetics* (Sharma, A.K. and Sharma, A., eds.). Gordon and Breach Science Publishers, London, pp. 79-104.

Vosa, C.G. (1997). Heterochromatin and ecological adaptation in Southern African *Ornithogalum* (Liliaceae). *Caryologia 50*: 97-103.

Watson, E.M. (1988). C-banding patterns in the Australian *Bulbine (Liliaceae)*: the annual group, *B. semibarbata s.* lato. *Plant Syst. Evol. 159*: 1-17.

Yamasaki, N. (1971). Karyotypanalyse an Hand des Färbungsmusters der Metaphasechromosomen von *Cypripedium debile* und *Trillium kamtschaticum*. *Chromosoma 33*: 372-381.

Zheng, J., Nakata, M., Uchiyama, H. and **Tanaka, R.** (1991). Giemsa C-banding patterns in several species of *Phaseolus* L. and *Vigna* Savi, Fabaceae. *Cytologia 56*: 459-466.

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CHROMOSOMA

High resolution physical mapping of 45S (5.8S, 18S and 25S) rDNA gene loci in the tomato genome using a combination of karyotyping and FISH of pachytene chromosomes

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Abstract. Karyotyping in combination with fluorescence in situ hybridization (FISH) on tomato pachytene chromosomes allowed identification and mapping of a major 45S (5.8S, 18S and 25S) rDNA site on the satellite of 2S and four minor loci, each at a proximal knob on 2L, 6S, 9S and 11S. Thus, the 45S rDNA loci are all located in heterochromatic regions. The five 45S sites are all transcriptionally active as evidenced by a maximum of ten nucleoli in meiotic cells at telophase or interphase. The 45S rDNA loci, as well as the 5S rDNA locus on 1S, were highlighted by chromomycin A_3, a GC-specific DNA ligand; this result is consistent with the high GC content of the rDNA genes. Satellite size varied dramatically between genotypes. Enzymatic maceration of tomato anthers followed by squashing in acetocarmine produced high quality chromosomal preparations and subsequent FISH images by reducing the strong autofluorescence inherent in the nucleolus and cytoplasm of tomato meiotic cells. Our protocol has potential in the construction of an integrated cytological, classical and molecular map of tomato.

Introduction

Fluorescence in situ hybridization (FISH) can contribute to physical mapping of DNA sequences in a genome. Pachytene chromosomes have several advantages over their somatic chromosome counterparts for such experiments. The more extended chromosomal DNA at the pachytene stage makes it easier for a probe to access the target sequence(s) in chromosome(s) and thus increases hybridization efficiency (Shen et al. 1987). The ability to resolve DNA target sites that are close together in a chromosomal subregion is also enhanced (Bello et al. 1989). In some plant species, such as tomato, a large number of meiotic cells at the pachytene stage can be readily obtained from a single anther.

A reliable chromosomal identification procedure that is compatible with FISH is essential in experiments aimed at mapping sequences to chromosome(s) or defined chromosomal regions. In plant genome studies, the general lack of cytogenetic markers and the presence of cell walls, cytoplasmic debris and strong autofluorescence in meiotic cells of some species (e.g., tomato, Xu and Earle 1996) all contribute to the limited success of physical mapping via FISH.

In this paper, we report the identification and accurate assignment of major and minor 45S (5.8S, 18S and 25S) rDNA gene loci to pachytene chromosome subregions in tomato using an improved procedure combining karyotyping and FISH. We find that the 45S rDNA loci are all located in heterochromatic regions and are all transcriptionally active. High quality chromosomal preparations for karyotyping and subsequent FISH images or chromomycin staining were obtained using the improved protocol. This approach has applications in high resolution physical mapping and in the determination of the chromatin origin of a DNA sequence or expressed gene in tomato.

Materials and methods

Chromosomal preparations. Two cultivars of tomato (*Lycopersicon esculentum* Mill.), Rio Grande and Motelle ($2n=2x=24$), were used. For meiotic studies, flower buds of plants grown in a greenhouse were fixed in ethanol:glacial acetic acid (3:1) for about 1 h. Anthers were examined under a phase contrast microscope to select buds at the pachytene stage. Anthers from the selected buds were washed with distilled water and macerated with an enzyme solution containing 2% cellulose (Worthington Biochemical Corporation, New Jersey) and 1% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo) in citrate buffer, pH 4.5 for 40–60 min at 37° C. The anthers were then washed twice in water and kept in water for about 1 h. Anthers were transferred onto a glass slide and gently tapped in a small drop of water to produce a cell suspension. A drop of 4% acetocarmine was added to and mixed with the cell suspension before squashing. At least ten meiotic cells

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with well-spread chromosomes were photographed for karyotyping each cultivar. Coverslips were removed, and the slides were destained in 45% acetic acid and incubated in 95% ethanol, for 5 min each, and then air-dried for several hours at room temperature.

Addition of iron acetate to the fixative is a common practice in the preparation of pachytene chromosomes of tomato because it intensifies the acetocarmine-stained chromosomal image (Ramanna and Prakken 1967); however, we found that it impairs subsequent FISH and therefore we did not include it in the fixative. Application of a few drops of fixative to the cell suspension during preparation of chromosomes after enzymatic maceration increases the proportion of cells with well-spread chromosomes and improve the efficiency of signal detection in subsequent FISH but results in a poor acetocarmine-stained chromosomal image, making karyotyping difficult.

Probe. The 45S rDNA plasmid was kindly provided by K. Arumuganathan, University of Nebraska. It contains a 9.1 kb insert isolated from tomato and cloned in the vector pUC18 by P. Palukaitis, Cornell University (Perry and Palukaitis 1990). The insert includes the 5.8S, 18S, and 25S rDNA subunits and nontranscribed spacer sequences. The plasmid was biotinylated using BioNick translation kit (GIBCO BRL).

Fluorescence in situ hybridization. The FISH procedure of Mukai et al. (1993) was used with the following modifications. Briefly, posthybridization washing was conducted at 40° C, and a high stringency wash (0.1XSSC) was used. Bovine serum albumin (3%) in 4XSSC was used for preincubation blocking and subsequent incubation of fluorescein isothiocyanate (FITC)-avidin or rhodamine-avidin D (Vector Laboratories). (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate.) Slides were washed in PN buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 0.1% Nonidet P-40, pH 8.0) for about 1 h and then mounted in Vectashield mounting medium (Vector Laboratories) containing propidium iodide (PI) or 4′,6-diamidino-2-phenylindole (DAPI). Slides were examined with an Olympus Vanox AHBT fluorescence microscope with single band pass filters or a triple band pass filter.

Biotinylated pUC18 alone was used in the FISH experiments as the negative control. No hybridization signals were seen in more than 100 meiotic cells examined for each cultivar. This confirms that there is no homology between the vector DNA and tomato chromosomes.

Silver staining and counting of nucleoli. To determine the activity of the 45S rDNA sites, silver staining and counts of nucleoli of meiotic cells were conducted on slides prepared using either a traditional squash method or a short (15 min) enzymatic maceration, which helped soften cell walls but did not dissolve the nucleoli. The silver-staining procedure of Howell and Black (1980) was used. Briefly, 2 drops of gelatin solution containing 2% (w/v) of gelatin and 1% (by vol.) of formic acid and 4 drops of $AgNO_3$ solution (50% w/v) were placed on chromosomal preparations. The slides were stained for approximately 2 min at 70° C, rinsed in distilled water, air-dried and mounted in Permount.

Chromomycin A_3/DAPI staining. Preparations were mounted overnight in a drop of Vectashield mounting medium containing chromomycin A3 solution (50 µg/ml). A drop of Vectashield mounting medium containing a mixture of chromomycin A3 (40 µg/ml) and DAPI (2 µg/ml) was then added. Observation and photography were conducted using a blue filter for chromomycin A_3 and a UV filter for DAPI.

Karyotyping. Each of the 12 bivalent chromosomes stained with acetocarmine was identified and labeled according to previously established karyotypes (Ramanna and Prakken 1967; Khush and Rick 1968). Presence of lightly stained (achromatic) or darkly stained (chromatic) regions along the length of each chromosome defined chromosomal landmarks such as telomeres or centromeres. Each centromere (small triangles in Fig. 1A) is recognized as a light-staining region flanked by two chromatic regions. It is generally accepted that the pericentric chromatic regions in tomato are heterochromatin (Khush et al. 1964; Xu and Earle 1996).

The 20 meiotic cells photographed after acetocarmine staining were photographed again, 10 after FISH and 10 after staining with chromomycin A3/DAPI (5 cells for each cultivar). Fuji color reprint film (ISO 1600) was used for photographs of FISH and Kodak color reprint film (ISO 1000), for photographs of chromomycin/DAPI staining.

A photograph of acetocarmine-stained pachytene chromosomes was copied onto a transparent sheet. The sheet was then superimposed on the photo of the same chromosomes stained with FISH or chromomycin A3/DAPI. This enabled an exact assignment of a FISH or fluorescent-dye stained signal to its chromosomal landmark.

Results

Karyotyping by acetocarmine staining

Enzymatic maceration of anthers followed by squashing in acetocarmine stain resulted in a high proportion of meiotic cells with well-spread chromosomes. Acetocarmine stainability of the pachytene chromosomes (Fig. 1A, C, E, G) was comparable to that obtained by the addition of iron in traditional fixation of anthers (Ramanna and Prakken 1967).

A spherical nucleolus to which the end [or nuclear organizer region (NOR)] of chromosome 2 was attached was always seen in tomato meiotic cells prepared by traditional procedures (Ramanna and Prakken 1967; Khush

Fig. 1. A–F Sequential acetocarmine staining (**A, C, E**) and fluorescence in situ hybridization (FISH) (**B, D, F**) of pachytene chromosomes allows accurate mapping of each 45S rDNA locus to a chromosomal subregion. FISH signals are tagged with fluorescein isothiocyanate (**B, F**) or rhodamine (**D**), and chromosomes are counterstained with propidium iodide (**B, F**) or 4′,6-diamidino-2-phenylindole (DAPI) (**D**). Centromeres, the major 45S rDNA site and minor sites are indicated with *small triangles*, and *large* and *small arrows*, respectively. (Short and long arms are indicated with *S* and *L*, respectively.) Note the variation in satellite size, with the satellite of cv. Rio Grande (**C, E**) being two to three times larger than that of cv. Motelle (**A, G**), as well as satellite shapes: diamond (**A**) or fork (**C, E**). A satellite gap (*double bars*) is clearly seen (**C, E**), and the gap along with the satellite is hybridized (**D, F**). **A, B** Each of the 12 bivalent chromosomes is identified and labeled (**A**), and FISH signals are located on 2S, 2L, 6S, 9S and 11S (**B**). **C, D** Part of a chromosomal spread showing the characteristic features of the proximal short arm of 2S with three heterochromatic knobs, corresponding to the three solid circles on the cytological map (Fig. 2). **E, F** A tiny hybridization signal at the proximal second heterochromatic knob on 2L is clearly visible in most cells. **G–L** Pachytene chromosomes of cv. Motelle sequentially stained with acetocarmine (**G, J**), chromomycin A3 (**H, K**) and DAPI (**I, L**) show that chromomycin A3 highlights all 45S loci and the 5S locus at the heterochromatic knob immediately adjacent to the centromere on 1S. **G–I** An entire cell and **J–L**, only a part (1S plus much of the proximal region of 1L) of chromosome 1 with the 5S site clearly seen (*small arrow*). Note the similarity in staining pattern with acetocarmine, chromomycin and DAPI with strong staining in proximal regions

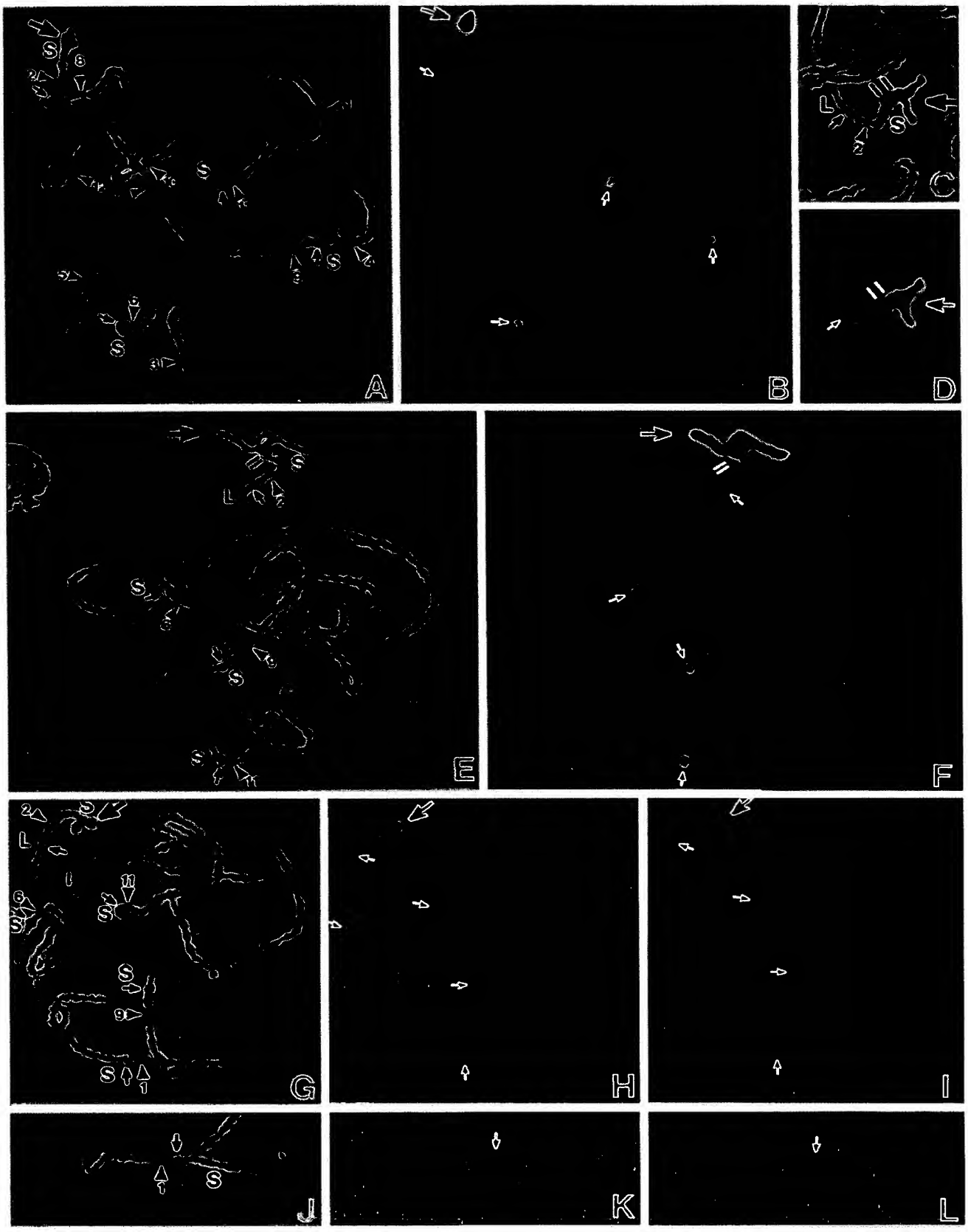

Fig. 1A–L

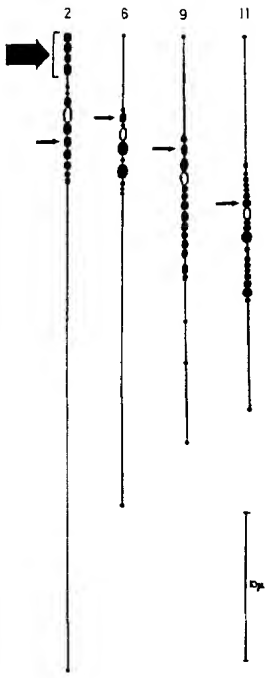

Fig. 2. Locations of 45S rDNA sites on the cytological map. The major site on 2S is marked with a *large arrow*, and the four minor sites on 2L, 6S, 9S and 11S are marked with *small arrows*. *Open circles* represent centromeres and *solid circles*, heterochromatic knobs. The original cytological map was constructed by Khush and Rick (1968) and is presented here with minor modifications

and Rick 1968; Xu and Earle 1996). After 40–60 min enzymatic maceration, the nucleolus disappeared, and a gap that defined the satellite and the distal end of the proximal arm of 2S was now clearly visible in some spreads of pachytene chromosome (double bars in Fig. 1C, E). The proximal arm of 2S had three heterochromatic knobs, as shown in Fig. 1C and illustrated as three solid circles in the cytological map (Fig. 2) (Khush and Rick 1968). Karyotypes of acetocarmine-stained pachytene chromosomes of cv. Motelle and cv. Rio Grande looked similar except for the size of the darkly stained satellite at the end of 2S (Fig. 1A versus 1E, see large arrows). The satellite of cv. Rio Grande was approximately two to three times longer than that of cv. Motelle. In spreads of pachytene chromosomes prepared with enzymatic maceration, some satellites had a fork shape (Fig. 1C, E) instead of the diamond (Fig. 1A) or bar shape also seen after use of direct squashing methods (Khush and Rick 1968; Ramanna and Prakken 1967; Xu and Earle 1996).

Fluorescence in situ hybridization

The 45S rDNA probe clearly hybridized to five regions in the tomato genome (Fig. 1B, F). Comparison of photographs of karyotypes and FISH of the same cells made it possible to localize each of the gene loci to a chromosomal region. The five 45S loci were all located in heterochromatic regions. The major site on 2S (large arrows in Fig. 1A–F) covered the entire satellite plus the gap (satellite stalk) between the proximal end of the satellite and the distal end of the proximal short arm, whenever this gap was visible (double bars in Fig. 1C–F). The four minor sites were all in the proximal regions: at the second knob on 2L, 6S, 9S, and the knob immediately adjacent to the centromere on 11S (small arrows in Fig. 1A, B, E, F). The hybridization signals on 6S, 9S and 11S

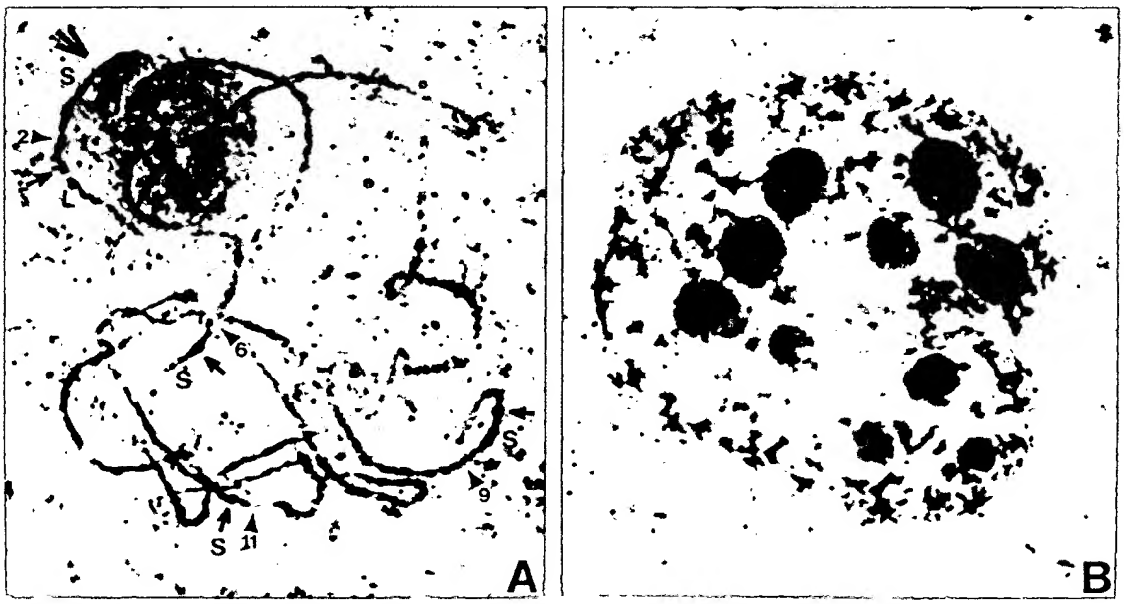

Fig. 3A, B. Silver-stained meiotic cells of cv. Motelle. **A** A spread of pachytene chromosomes showing many darkly stained pericentric heterochromatic regions. The 45S sites are indicated with a *large arrow* (major site) and *small arrows* (minor sites). The centromeres of relevant chromosomes are indicated with *triangles*. **B** A meiotic cell at interphase showing ten nucleoli, indicating that the five 45S rDNA sites in the tomato genome are all active

were approximately the same size and three to four times larger than that on 2L (Fig. 1E, F). The size of the hybridization signal on each minor site looked similar to that of its heterochromatic knob. In cv. Motelle the four minor sites comprised approximately 50% of the total length of the chromosome hybridizing with the 45S rDNA genes. In cv. Rio Grande the minor sites occupied a smaller proportion (30–40%), because the satellite was larger in that genotype. Figure 2 illustrates the locations of the gene loci in the cytological map.

Silver staining and counting of nucleoli

Silver stained darkly many of the pericentric heterochromatic regions of pachytene chromosomes, giving rise to a staining pattern similar to that seen with acetocarmine staining (Fig. 3A). This made gross identification of individual chromosomes possible. The 45S sites (arrows in Fig. 3A) were among the darkest regions. Silver stained heterochromatic regions in addition to the 45S sites, as also noted in other species, including Algerian hedgehog (Sanchez et al. 1995) and rye (Murray 1994); for this reason silver staining alone could not be used to determine the activity or inactivity of a 45S site in the tomato genome.

A maximum of ten nucleoli was readily seen in some meiotic cells at telophase or interphase (Fig. 3B). This result indicated that the five 45S sites revealed by FISH were involved in formation of nucleoli and, therefore, were all active.

Chromomycin A_3/DAPI staining

Chromomycin A_3 stained very brightly at all 45S sites and also at the 5S rDNA locus at the proximal heterochromatic knob on 1S (arrows in Fig. 1G–L). The 5S rDNA locus was previously identified using FISH (Xu and Earle 1996). The size or intensity of each region highlighted with Chromomycin A_3 approximated that of the corresponding FISH signal. The tiny site on 2L appeared very weak in observation or on photographs (Fig. 1H) because it was extremely vulnerable to rapid fading of chromomycin A_3 fluorescence. The intensity of chromomycin A_3 staining at the 5S locus varied between the two genotypes, with cv. Rio Grande having a much weaker signal. All sites that were stained strongly with chromomycin A_3 were weakly stained with DAPI (Fig. 1H versus I). In addition, chromomycin A_3 and DAPI (also PI, see Fig. 1B, F) showed similar staining patterns in many of the chromosomal regions with stronger staining in proximal heterochromatic regions and telomeres.

Discussion

We demonstrate success in the high resolution physical mapping of the five 45S rDNA loci in the tomato genome, especially in assigning each of the four minor sites to a proximal heterochromatic knob in a specific chromosome. Previously, Vallejos et al. (1986) mapped the 45S rDNA genes of tomato to a single locus in the terminal position of 2S by using a pea genomic clone of 45S rDNA and restriction fragment length polymorphisms (RFLPs) between *Lycopersicon pennelli* and *L. esculentum*. Ganal et al. (1988) later estimated the copy number of the 45S rDNA in the tomato genome at about 2300 copies and observed the single distal location of the genes, using tomato genomic sequences homologous to pea 45S genes and in situ hybridization with an enzyme-mediated detection system. Using a direct FISH procedure on somatic chromosomes and well-characterized 45S rDNA genes isolated from tomato (Perry and Palukaitis 1990), Xu and Earle (1994) observed that six chromosomes (presumably three pairs) had a minor site in an interstitial region in addition to the previously mapped major site on 2S. In that work four tomato genotypes were examined, including cv. VF36 used by Vallejos et al. (1986) and Ganal et al. (1988) in their mapping studies. Our current work identified an additional minor site and revealed the chromosomal location of each minor site. Failure of the earlier workers to detect the minor 45S rDNA sites may be due to the absence of the minor site sequence(s) in the heterologous probe from pea or to a limited number of RFLPs detected in the proximal regions.

In preparations made by using traditional protocols, the nucleolus and cytoplasm of tomato somatic cells (Xu and Earle 1994) and meiotic cells (Xu and Earle 1996) show strong red or yellow autofluorescence. This is a serious handicap in FISH for it interferes with the hybridization signals tagged with the commonly used fluorochromes, fluorescein or rhodamine. In preparations using our (40–60 min) enzymatic maceration procedure, spherical nucleoli disappeared, and the overall intensity of autofluorescence of the cytoplasm was reduced. Removal of the nucleolus also enables the visualization of the gap on 2S that separates the satellite and the distal end of the proximal short arm in the pachytene chromosome. This allows an accurate determination of satellite size and the chromosomal segment with the 45S genes. In traditional protocols, association of the darkly stained nucleolus with much of 2S makes the gap barely visible (Khush and Rick 1968).

DAPI (preferentially staining AT-rich DNA sequences), chromomycin A_3 (for GC-rich sequences), PI (general stain for DNA, see Fig. 1B, F), and acetocarmine all showed similar staining patterns on pachytene chromosomes, with stronger staining in proximal heterochromatic regions (or knobs) and telomeres. Recently, Peterson et al. (1995) estimated that the amount of DNA per unit length of tomato pachytene chromosomes is about six times greater in heterochromatic regions than in euchromatic regions. The strong correlation of DAPI and PI with acetocarmine staining patterns indicates that DAPI and PI staining could be alternatives for gross identification of tomato pachytene chromosomes after FISH although they may be less efficient in recognizing detailed chromosomal landmarks than acetocarmine staining before FISH.

Visualization of 45S and 5S rDNA gene loci by chromomycin A3 staining agrees with the previous data

on the high GC content in these genes. The GC content in the coding regions is about 59% for 45S rDNA (Kiss et al. 1988) and 55% for 5S rDNA (Vandenberghe et al. 1984), while it is only 37% for the tomato genome overall (Messeguer et al. 1991). Knowledge of the extremely high copy number of 45S rDNA on tomato 2S and the high GC content of 45S rDNA was exploited in flow cytometry aimed at construction of a chromosome 2-specific library (Arumuganathan et al. 1994).

Several maps of tomato have been constructed, including a classical map of traits (Khush and Rick 1968), a cytological map of detailed landmarks of pachytene chromosomes (Khush and Rick 1968; Ramanna and Prakken 1967), a physical map of crossover frequency on synaptonemal complexes (Sherman and Stack 1995), and a molecular map highly saturated with DNA markers (Tanksley et al. 1992). Long, single copy DNA probes such as yeast or bacterial artificial chromosome (YAC or BAC) sequences (about 80–100 kb in size) are currently being developed and can be used with our protocol for karyotyping and FISH of pachytene chromosomes. This would enable the construction of an integrated molecular, cytological and classical map of tomato.

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References

Arumuganathan K, Martin GB, Telenius H, Tanksley SD, Earle ED (1994) Chromosome 2-specific DNA clones from flow-sorted chromosomes of tomato. Mol Gen Genet 242: 551–558

Bello MJ, Salagnon N, Rey JA, Guichaoua MA, Berge Lefranc JL, Jordan BR, Luciani JM (1989) Precise in situ localization of NCAM ETS1 and D11S29 on human meiotic chromosomes. Cytogenet Cell Genet 52: 7–10

Ganal MW, Lapitan NLV, Tanksley SD (1988) A molecular and cytogenetic survey of major repeated DNA sequences in tomato (*Lycopersicon esculentum*). Mol Gen Genet 213: 262–268

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014

Khush GS, Rick CM (1968) Cytogenetic analysis of the tomato genome by means of induced deficiencies. Chromosoma 23: 452–484

Khush GS, Rick CM, Robinson RW (1964) Genetic activity in a heterochromatic chromosome segment of the tomato. Science 25: 1432–1434

Kiss T, Kis M, Abel S, Solymosy F (1988) Nucleotide sequence of the 17S-25S spacer region from tomato rDNA. Nucleic Acids Res 16: 7179

Messenguer R, Ganal MW, Steffen JC, Tanksley SD (1991) Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. Plant Mol Biol 16: 753–770

Mukai Y, Nakahara Y, Yamamoto M (1993) Simultaneous discrimination of the three genomes in hexaploid wheat by multicolor fluorescence in situ hybridization using total genomic and highly repeated DNA probes. Genome 36: 489–494

Murray BG (1994) Heterochromatin and silver banding of rye (*Secale cereale*, Gramineae) chromosomes. Plant Syst Evo 193: 243–248

Perry KL, Palukaitis P (1990) Transcription of tomato ribosomal DNA and the organization of the intergenic spacer. Mol Gen Genet 221: 102–112

Peterson DG, Stack SM, Price HJ, Johnston JS (1995) Distribution of DNA in heterochromatin and euchromatin of *Lycopersion esculentum* pachytene chromosomes. Tomato Genet Coop Rep 45: 35

Ramanna MS, Prakken R (1967) Structure of and homology between pachytene and somatic metaphase chromosomes of the tomato. Genetica 38: 115–133

Sanchez A, Jimenez R, Burgos M, Stitous S, Zurita F, Diaz De LaGuardia R (1995) Cytogenetic peculiarities in the Algerian hedgehog: silver stains not only NORs but also heterochromatic blocks. Heredity 75: 10–16

Shen DL, Wang ZF, Wu M (1987) Gene mapping on maize pachytene chromosomes by in situ hybridization. Chromosoma 95: 311–314

Sherman JD, Stack SM (1995) Physical map of crossover frequency on synaptonemal complexes from tomato primary microsporocytes. Tomato Genet Coop Rep 45: 42–43

Tanksley SD, Ganal MW, Prince JP, de Vicente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni JJ, Grandillo S, Martin GB, Messeguer R, Miller JC, Miller L, Paterson AH, Pineda O, Roder MS, Wing RA, Wu W, Young ND (1992) High density molecular maps of the tomato and potato genomes. Genetics 132: 1141–1160

Vallejos CE, Tanksley SD, Bernatzky R (1986) Localization in tomato genome of DNA restriction fragments containing sequences homologous to the rDNA (45S), the major chlorophyll a/b binding polypeptide and the ribulose bisphosphate carboxylase genes. Genetics 112: 93–105

Vandenberghe A, Chen M, Dams E, de Baere R, de Roeck E, Huysmans E, de Wachter R (1984) The correct nucleotide sequences of 5 S RNAs from six angiosperms with some notes on 5 S RNA secondary structure and molecular evolution. FEBS Lett 171: 17–23

Xu J, Earle ED (1994) Direct and sensitive fluorescence in situ hybridization of 45S rDNA on tomato chromosomes. Genome 37: 1062–1065

Xu J, Earle ED (1996) Direct FISH of 5S rDNA on tomato pachytene chromosomes places the gene at the heterochromatic knob immediately adjacent to the centromere of chromosome 1. Genome 39: 216–221

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Replicon clusters may form structurally stable complexes of chromatin and chromosomes

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SUMMARY

Nuclear DNA replication was monitored 'in situ' in pea nuclei with the bromodeoxyuridine antibody technique. The labelling appeared to be restricted to a number of finely distinct spots. The labelling was followed through three subsequent cell cycles in meristematic and differentiating pea root cells. The results show that the spots as seen just after the labelling persist distinctly over the mitotic chromosomes as well as in the nuclei of the following cell cycles up to 44 hours after the pulse. Moreover, they are also present in the nuclei of differentiating cells. The spots over the mitotic chromosomes in specific cases give rise to a dynamic banding. Nuclei of the second and third cycle show absence of labelling in specific zones, owing to the segregation of the labelled strands of chromosomal DNA. The maintenance of the spotted appearance of the replication clusters through all stages of the three subsequent cell cycles may be an indication in favour of the hypothesis that such clusters represent structurally stable replicon complexes held together by the nuclear matrix and the chromosome scaffold.

Key words: DNA replication, immunofluorescence, bromodeoxyuridine labelling, cell cycle, chromatin structure

INTRODUCTION

DNA replication in eukaryotic cell nuclei is achieved through the simultaneous activation of a number of groups of adjacent replicons called replicon clusters (Huberman and Riggs, 1968). Analysis of the temporal and spatial order in which such replicon clusters are activated has led to the conclusion that it is not a random process. Early results obtained through autoradiography of [^{3}H]thymidine-labelled nuclei showed that heterochromatin replicated at the end of the S-phase and was localized at the nuclear periphery (Ockey, 1972; Comings and Okada, 1973; Sparvoli et al., 1976). In especially favourable cases it was possible to show that such labelling appeared as a defined number of spots located at the nuclear periphery, which corresponded to specific heterochromatic segments of the chromosomes (Sparvoli et al., 1977).

The limitation imposed by the low resolution level of autoradiographs of [^{3}H]thymidine-labelled DNA was overcome by immunofluorescence detection of incorporated bromodeoxyuridine (BrdUrd) or biotin-labelled dUTP. In fact this technique allowed a much finer detection of the sites of DNA replication (Nakamura et al. 1986; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; O'Keefe et al., 1992; Cox and Laskey, 1991). As a consequence it appeared that the incorporated BrdUrd or biotinylated dUTP was not distributed uniformly over the S-phase nuclei, but appeared as a number of finely distinct spots or foci that vary in number and location at different moments of the S-phase. Such foci have been interpreted, by some authors, as localized simultaneous replication of a large number of tandemly arranged replicons held together by the elements of a 'nuclear matrix' (Cook, 1988; Nakayasu and Berezney, 1989; Jackson, 1990; Hozák et al., 1993).

Nevertheless, since the real presence of a nuclear matrix has often been challenged, other authors have proposed different interpretations (Adachi and Laemmli, 1992). The different ways of interpreting the observed foci have meaningful consequences regarding the model of DNA organization in the nucleus and its method of replication.

On the basis of such contrasting hypotheses, taking advantage of the pattern of plant growth and development, we devised an experimental protocol that could provide some evidence that might help in discriminating between these different interpretations. We showed previously that when pea roots are fed with a pulse of BrdUrd, the incorporated precursor can be detected in nuclei by immunofluorescence (Levi et al., 1987) and, also in this plant material, the labelling appears in the form of distinct spots (Levi et al., 1990). In the root tip, the meristematic cells undergo a series of cell cycles and their derivatives, in a more proximal zone, can stop dividing and start to differentiate. After a brief pulse with BrdUrd, it is then possible to follow the fate of the labelled DNA replication sites in interphase nuclei and mitotic chromosomes in the intact organism throughout subsequent cell cycles and in differentiating cells. The evidence that we obtained shows that the replicon clusters appear to form stable complexes. This finding

is interpreted as being in agreement with the hypothesis that replicon clusters are held together by the nuclear matrix.

MATERIALS AND METHODS

Three-day-old seedlings of *Pisum sativum* L. var. Lincoln (Ingegnoli, Italy), grown on Agriperlite (Superlite, Vic Italiana, Italy) at 25°C in the dark were used.

The seedlings were directly pulsed with BrdUrd (asynchronous labelling) or synchronized with hydroxyurea (HU) in order to label the nuclei in specific moments of the S-phase (Fig. 1).

Synchronization

Seedlings were transferred with the root immersed in an aerated bath containing 2.5 mM HU for 12 hours in the dark at 25°C, and then washed thoroughly and transferred to aerated distilled water. The cell cycle kinetics of the recovery was monitored by flow cytometry (Fig. 1) (Levi et al., 1992).

BrdUrd labelling

The seedlings were transferred with the roots immersed in an aerated solution of BrdUrd (100 μM) for 30 minutes at 25°C in the dark. At the end of the pulse, the roots were rinsed and fixed immediately, or the seedlings were transferred for the chase period in distilled water. In some experiments, after 3 hours of chase with distilled water, the seedlings were transferred in 2 mM 8-quinolinol, a microtubule inhibitor, in order to accumulate more mitotic figures.

Fixation and slide preparation

At the proper time, root apices (0-2 mm) or differentiating root segments (2-5 mm from the tip) were fixed in 4% formaldehyde in Tris buffer (Levi et al., 1986) and then rinsed thoroughly in buffer. Nuclei were extracted by crushing the tissue with a glass rod, and nuclear smears were prepared as described previously (Levi et al., 1986). The slides were stored without problems up to several weeks

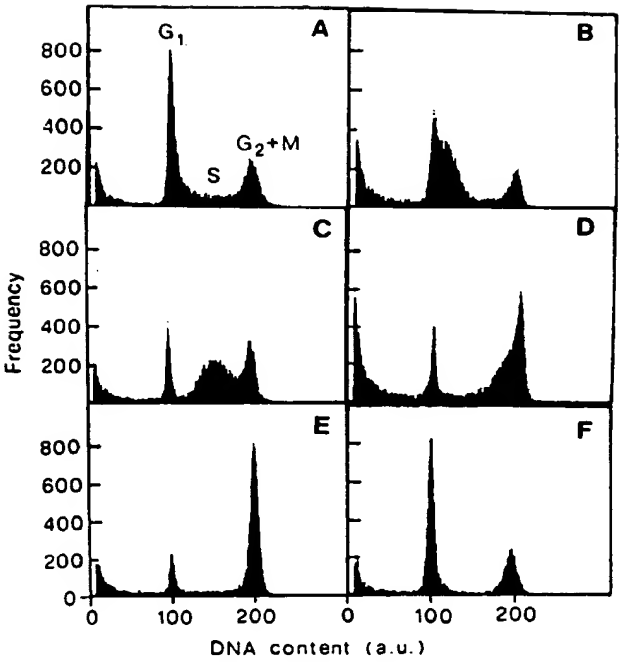

Fig. 1. Synchronization of the cell cycle in 3-day-old pea seedlings with HU, monitored by flow cytometry. a.u., arbitrary units. (A) Unsynchronized control; (B) after the release from 12 hours of HU treatment; (C,D,E,F) 2, 3, 4 and 8 hours later, respectively. Note the exchange between the height of G_1 and G_2+M peaks in E and F, due to cell division.

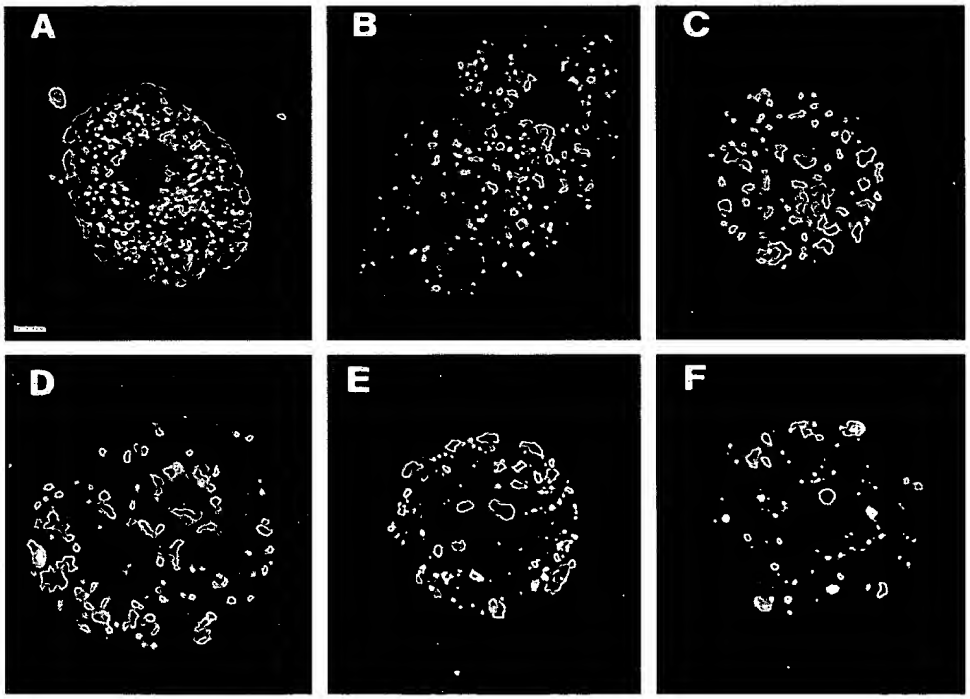

Fig. 2. Appearance of BrdUrd immunofluorescence localization immediately after the BrdUrd pulse. Bar, 5 μm. The BrdUrd is localized in a number of tiny spots whose number and localization show these basic patterns: pattern 1 (A,B) shows 200-300 spots distributed all over the nucleus and should be ascribed to the early S-phase. Pattern 2 (C,D) is characterized by a typical labelling around the nucleolus and a preferential distribution of the rest of the labelling around the nuclear periphery and should be ascribed to middle/late S-phase. Pattern 3 (E,F) belongs to the late S-phase and shows that most of the labelling is found at the nuclear periphery. Note in D,E,F the presence of spots that appear larger and in the form of curved or horse-shoe-shaped lines.

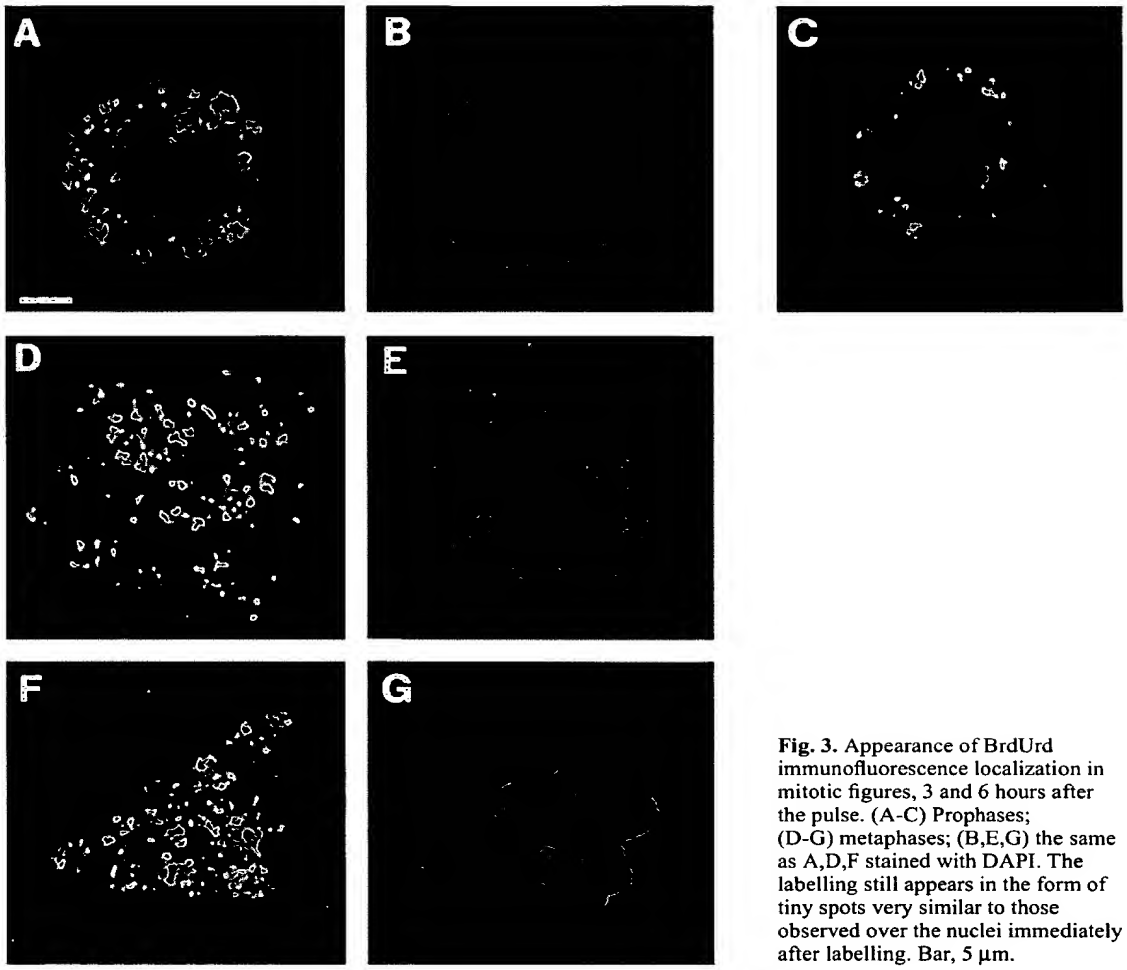

Fig. 3. Appearance of BrdUrd immunofluorescence localization in mitotic figures, 3 and 6 hours after the pulse. (A-C) Prophases; (D-G) metaphases; (B,E,G) the same as A,D,F stained with DAPI. The labelling still appears in the form of tiny spots very similar to those observed over the nuclei immediately after labelling. Bar, 5 μm.

in absolute ethanol at −20°C. A sample of the suspension of nuclei was stained with 4′,6-diamidino-2-phenylindole (DAPI) and used for flow cytometric analysis (Levi et al., 1992).

Immunofluorescent staining

The slides stored in ethanol were immersed for 30 minutes in methanol at room temperature, air dried, rehydrated for a few minutes in Tris buffer and hydrolysed for 1 hour at 25°C in 2 M HCl. The immunofluorescent staining was performed essentially as described previously (Levi et al., 1987, 1990), with an indirect method: monoclonal anti-BrdUrd antibody (Becton Dickinson), biotinylated secondary antibody and Texas Red-streptavidin. Slides were counterstained with DAPI and mounted in Tris buffer for immediate observation and photographing, or dried and conserved unmounted at 4°C in the dark for a few days. The nuclei and stain remained well preserved. Slides were examined with a Zeiss Axioplan fluorescence microscope and photographed with Kodak T-Max 400 professional film.

RESULTS

Patterns of labelling of S-phase nuclei

After a 30 minute pulse of BrdUrd, the nuclei always show a distribution of labelling in the form of finely distinct spots or foci whose number and localization appear to conform to three basic patterns (Fig. 2), which show some similarities with those found in mammalian cells (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992).

The first pattern (Fig. 2A,B) appears as a very large number of finely distinct small spots (we counted an average of 352 spots per nucleus, with a minimum of 210 and a maximum of 410). Their main characteristic is to appear to be distributed throughout the nucleus except for the nucleolar zone. This kind of pattern is very similar to type I described by Nakayasu and Berezney (1989) and to pattern 1 described by O'Keefe et al. (1992), and should be ascribed to the early S-phase.

The second distinct pattern (Fig. 2D) is characterized by the presence of a ring of spots around the nucleolar zone in addition to a limited number of spots that appear distributed prevalently around the nuclear periphery. This pattern is similar to Nakayasu and Berezney's (1989) type II and to O'Keefe's (1992) pattern 3. The pattern shown in Fig. 2C could be intermediate between those in A,B and that in D.

The third pattern represented in Fig. 2E,F shows a moderate

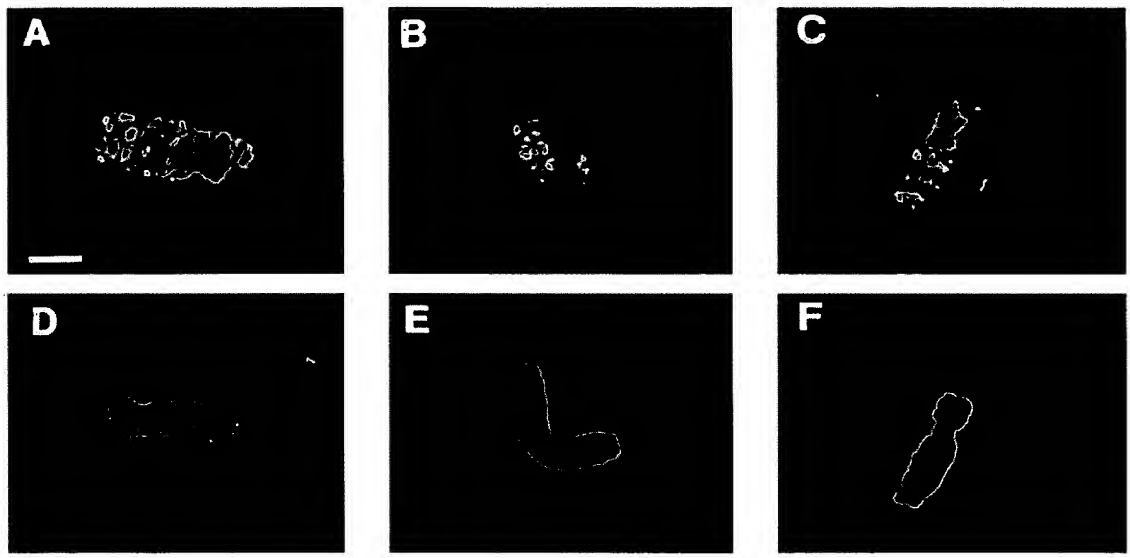

Fig. 4. Appearance of BrdUrd immunofluorescence in metaphasic chromosomes 6 hours after the pulse. It can be seen that the single chromosomes also show the typical spotted appearance. (D,E,F) DAPI. Bar, 5 μm.

number of spots preferentially located at the nuclear periphery and corresponding to the distribution of heterochromatin. This last pattern in our opinion corresponds to Nakayasu and Berezney's (1989) type III and to O'Keefe's (1992) patterns 4 and 5 and should be ascribed to the late S-phase. That this is the case is also confirmed by their high frequency in cells labelled at the end of the S-phase after synchronization with HU. In the micrographs of the last two stages a number of spots appear to be larger, with curved or horseshoe shapes as is also observed in mammalian cells.

Patterns of labelling of mitotic chromosomes

If the root tips are fixed 3 to 6 hours after a late S-phase pulse (Fig. 1) and the nuclei extracted and spread as described in Materials and Methods it is possible to find several labelled prophases and a number of group or single metaphasic chromosomes (because of the extraction procedure and of spreading, it is very difficult to obtain complete metaphasic spreads). Fig. 3A,C show the appearance of the labelling of two prophasic nuclei in which the nucleus in Fig. 3C corresponds to a cell labelled at the end of S-phase. As can be seen, the chromosomal portions in the focal plane exhibit a number of finely distinct spots similar to those observed in the S-phase nuclei. The spotted appearance of chromosomes is most evident in metaphasic chromosomes either when they form clusters as in Fig. 3D,F or when they are observed as single chromosomes as shown in Fig. 4. Fig. 5, moreover, shows a gallery of metaphasic chromosomes that correspond to nuclei labelled at the end of the S-phase. They exhibit the clear presence of 'replication banding', each band is made up of two or more spots. It can be observed that such bands occur mostly in the pericentromeric regions, and in secondary constrictions and satellites. All the mitotic chromosomes, then, show labelling made up of a number of distinct spots similar to those observed in S-phase nuclei and these spots, in favourable conditions, appear to determine a replication banding.

Nuclei labelling patterns 20 and 44 hours after the pulse

Fig. 6 shows a number of different labelled nuclei as they appear 20 or 44 hours after the BrdUrd pulse. Fig. 6A,B represent a nucleus from the root tip (0-2 mm zone), while the nuclei from 6C to 6H are from the 2-5 mm zone, where a relevant number of nuclei have ceased dividing because their cells have started the differentiation process. As can be seen, all the nuclei, which are representative of the population of labelled nuclei, show a typical spotted appearance similar to that already encountered in the previous stages. Nevertheless, most of them (Fig. 6A,C,E) show that the labelling is restricted to specific well delimited areas. Some of the nuclei, like the one in Fig. 6G, do not exhibit labelling distributed to specific areas but have a uniformly spotted appearance. We believe that these nuclei may come from cells that ceased dividing and started the differentiation process. The elongated appearance of the nucleus in Fig. 6G is in agreement with this interpretation. Whether this interpretation is correct or not, all the labelled nuclei in the 2-5 mm zone show the spots and, according to our cytometric analyses, a relevant number of them should belong to cells that ceased dividing and initiated the differentiation process.

DISCUSSION

The first result of our study on the spatial and temporal distribution of DNA replication in plant cell nuclei is that it occurs in a number of distinct foci that show a close similarity to those observed in mammalian cells. Moreover, they show three distinct patterns of distribution of foci that have some basic features similar to those described for mammalian cells (Nakayasu and Berezney, 1989; O'Keefe et al., 1992). This similarity between completely unrelated organisms may

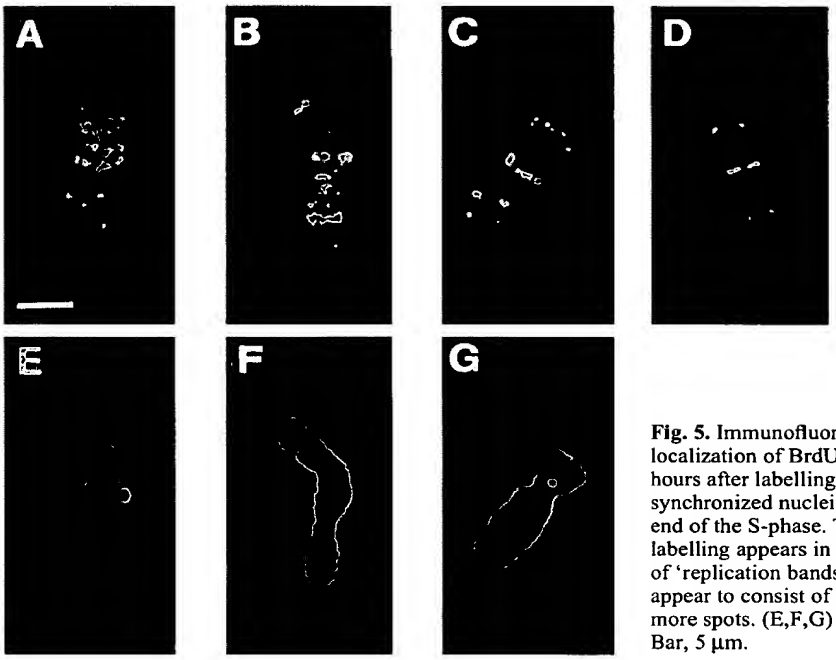

Fig. 5. Immunofluorescence localization of BrdUrd 3 or 6 hours after labelling of HU-synchronized nuclei at the end of the S-phase. The labelling appears in the form of 'replication bands' that appear to consist of two or more spots. (E,F,G) DAPI. Bar, 5 μm.

underlie some basic feature of the regulation of the temporal and spatial order of DNA replication in eukaryotes. Concerning this, we previously reported that the auxiliary protein of DNA polymerase δ, PCNA (proliferating cell nuclear antigen), is also distributed in distinct foci in pea nuclei when revealed by immunofluorescence with PC10 monoclonal antibody (Citterio et al., 1992). A speckled distribution was also found with an antibody against a sequence of the human topoisomerase II (Levi et al., 1994).

The appearance of BrdUrd labelling in S-phase nuclei in the form of distinct foci has been interpreted by various authors as reflecting the simultaneous localized activation of different clusters of replicons. Although there is a general agreement on the interpretation of such foci as representing the simultaneous activation of the various clusters of replicons, there is no agreement about the mechanism underlying this localized replication. As mentioned above, some authors (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Jackson, 1991) suggest that the observed foci are the consequence of permanent anchoring of the clusters of replicons to a nuclear matrix. This interpretation would be in agreement with the hypothesis that replication complexes are fixed on the nuclear matrix and DNA is spooled through it while it is being replicated (Cook, 1991). That this may be the case is firmly supported by the evidence presented by Hozák et al. (1993), who were able to visualize what they call 'replication factories' attached to the nucleoskeleton, which appear to be the sites where DNA replication occurs.

Our studies, addressed to follow the fate of the replicon clusters from the S-phase to the following stages of mitosis and the subsequent cell cycles up to 44 hours after the pulse, are in agreement with this hypothesis. In fact from our results it is possible to infer that the replicon clusters may form permanent complexes of chromatin and chromosomes.

The first indication comes from the chromosomes as they are observed during the first mitosis immediately following the BrdUrd pulse (Figs 4, 5). When the labelled chromosomes are submitted to close analysis they show that the label is distributed in a number of tiny spots very similar to the foci observed in S-phase nuclei and in G_2 nuclei, in apparent continuity with them. The possibility that the spots present in the mitotic chromosomes may correspond to the foci observed after the pulse is reinforced by the clearly spotted appearance of the replication bands that correspond to the late replicating DNA.

To ascertain whether the spots are a permanent feature of chromatin and chromosomes, we examined the nuclei 20 and 44 hours after labelling. In both cases most of the cycling cells should have gone through at least one or two cycles after the labelling, since the duration of the cell cycle in peas lasts about 14 hours (Van't Hof, 1974). As shown in Fig. 6, all nuclei show a distribution of label in the form of tiny spots, even if most of them show the labelling restricted to specific distinct areas.

All these findings, which are in agreement with the preliminary results obtained by Meng and Berezney (1991), can be explained assuming that the foci observed immediately after labelling and those present in the chromosomes and nuclei in the subsequent stages are the same. Therefore, if the foci represent the replicon clusters, these are structurally stable complexes.

This general picture shows several aspects that strengthen its significance and add particular points of interest.

The first element to be considered concerns the spots present in the mitotic chromosomes. In fact, if they correspond to the clusters of replicons, we have to consider that they are most probably held together by the chromosome scaffold. If this is the case, it seems plausible that the anchoring scaffold should be preceded, in the interphasic nucleus, by some similar structure, which could be the postulated nuclear matrix.

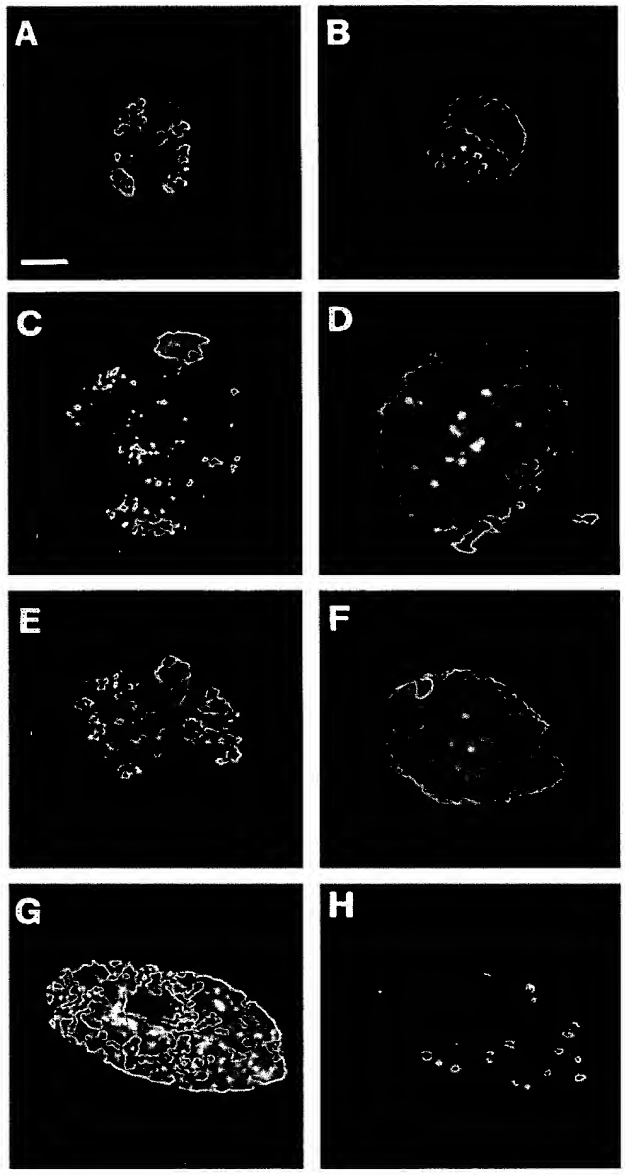

Fig. 6. This is a gallery of nuclei that were labelled with BrdUrd and then chased for 20 or 44 hours. Bar, 5 μm. (A) 20 hours, 0-2 mm zone; (C and E) 44 hours, 2-5 mm zone, show that the spots are restricted to specific zones, while definite territories appear without labelling. (G) 20 hours, 2-5 mm zone, is interpreted as a nucleus of a cell that arrested its cell cycle and started differentiation. (B,D,F,H) DAPI.

A further point of interest is the replication banding of chromosomes, which we were able to see clearly in relation to late-replicating DNA. Such bands are made up of contributions from two or more spots. This adds further support to the idea that the banding of chromosomes is also related to structural factors (Vogel et al., 1989; Manuelidis and Chen, 1990).

As a last point, we would like to mention the patterns of labelling shown by the nuclei 20 or 44 hours after the pulse. As we have shown, very often this is restricted to specific and defined areas, which can be explained by the segregation of the labelled strands in different nuclei as a consequence of semi-conservative replication of DNA and is in agreement with the findings that show that interphasic chromosomes occupy specific areas (Heslop-Harrison and Bennet, 1990).

We find it difficult to explain all these different findings concerning the labelling patterns without assuming the presence of specific structures that hold the replication clusters in place and cause them to appear as permanent structural complexes. In conclusion, we believe that the evidence presented here may be explained if the presence of an anchoring structure that could consist of the nuclear matrix and a chromosome scaffold is assumed.

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REFERENCES

Adachi, Y. and Laemmli, U. K.. (1992). Identification of nuclear pre-replication centers poised for DNA synthesis in Xenopus egg extracts: immunolocalization study of replication Protein A. *J. Cell Biol.* **119**, 1-15.

Citterio, S., Sgorbati, S., Levi, M., Colombo, B.M. and Sparvoli, E. (1992). PCNA and total nuclear protein content as markers of cell proliferation in pea tissue. *J. Cell Sci.* **102**, 71-78.

Comings, D. E. and Okada, T. A. (1973). DNA replication and the nuclear membrane. *J. Mol. Biol.* **75**, 609-618.

Cook, P. R. (1988). The nucleoskeleton: artefact, passive framework or active site? *J. Cell Sci.* **90**, 1-6.

Cook, P. R. (1991). The nucleoskeleton and the topology of replication. *Cell* **66**, 627-635.

Cox, L. S. and Laskey, R. A. (1991). DNA replication occurs at discrete sites in pseudonuclei assembled from purified DNA in vitro. *Cell* **66**, 271-275.

Fox, M. H., Arndt Jovin, D. J., Baumann, P. H. and Robert-Nicoud, M. (1991). Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblast. *J. Cell Sci.* **99**, 247-253.

Heslop-Harrison, J. S. and Bennet, M. D. (1990). Nuclear architecture in plants. *Trends Genet.* **6**, 401-405.

Hozák, P., Bassim Hassan A., Jackson, D. A. and Cook, P. R. (1993). Visualization of replication factories attached to a nucleoskeleton. *Cell* **73**, 361-373.

Huberman, J. A. and Riggs, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J. Cell Biol.* **32**, 327-341.

Jackson, D. A. (1990). The organization of replication centers in higher eukaryotes. *BioEssays* **2**, 87-89.

Jackson, D. A. (1991). Structure-function relationship in eukaryotic nuclei. *BioEssays* **1**, 1-10.

Levi, M., Tarquini, F., Sgorbati, S. and Sparvoli, E. (1986). Determination of DNA content by static cytofluorimetry in nuclei released from fixed plant tissue. *Protoplasma* **132**, 164-68.

Levi, M., Sparvoli, E., Sgorbati, S. and Chiatante, D. (1987). Rapid immunofluorescent determination of cells in the S phase in pea root meristems: an alternative to autoradiography. *Physiol. Plant* **71**, 68-72.

Levi, M., Sparvoli, E., Sgorbati, S. and Chiatante, D. (1990). Biotin-streptavidin immunofluorescent detection of DNA replication in root meristems through BrdUrd incorporation: cytological and microfluorimetric application. *Physiol. Plant* **79**, 231-235.

Levi, M., Pasini, E., Brusa, P., Chiatante, D., Sgorbati, S. and Sparvoli, E. (1992). Culture of pea embryo axes for studies on the reactivation of the cell cycle at germination. *In Vitro Cell Dev. Biol.* **28**, 20-24.

Levi, M., Sparvoli, E. and Corbetta, N. (1994). An antibody against a sequence of human topoisomerase II gives different immunofluorescence patterns in quiescent and proliferating nuclei of Pisum sativum L. *J. Exp. Bot.* **45**, 1157-1162.

Manuelidis, L. and Chen, T. L. (1990). A unified model of eukaryotic chromosomes. *Cytometry* **11**, 8-25.

Meng, C. and Berezney, R. (1991). Replication cluster domains persist throughout the cell cycle of mouse 3T3 cells. *J. Cell Biol.* **115**, 95a.

Nakamura, H., Morita, T. and Sato, C. (1986). Structural organization of replicons domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* **165**, 291-297.

Nakayasu, H. and Berezney, R. (1989). Mapping replicational sites in the eukaryotic cell nucleus. *J. Cell Biol.* **108**, 1-11.

Ockey, C. H. (1972). Distribution of DNA replicator sites in mammalian nuclei. II. Effect of prolonged inhibition of DNA synthesis. *Exp. Cell Res.* **70**, 203-213.

O'Keefe, R. T., Henderson, S. C. and Spector, D. L. (1992). Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. *J. Cell Biol.* **116**, 1095-1110.

Sparvoli, E., Galli, M. G., Mosca, A. and Paris, G. (1976). Localization of DNA replicator sites near the nuclear membrane in plant cells. *Exp. Cell Res.* **97**, 74-82.

Sparvoli, E., Galli, M. G., Chiatante, D. and Sgorbati, S. (1977). Perinuclear DNA and heterochromatin. *Exp. Cell Res.* **110**, 315-321.

van Dierendonck, J. H., Keyzer, R., van de Velde, C. J. H. and Cornelisse, C. J. (1989). Subdivision of S-phase by analysis of nuclear 5-bromodeoxyuridine staining patterns. *Cytometry* **10**, 143-150.

Van't Hof, J. (1974). The regulation of cell division in higher plants. *Brookhaven Symp. Biol.* **25**, 152-165.

Vogel, W., Autenrieth, M and Mehnert, K. (1989). Analysis of chromosome replication by BrdUrd antibody technique. *Chromosoma* **98**, 335-341.

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NOTE

Sequential chromosome banding and in situ hybridization analysis[1]

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JIANG, J., and GILL, B.S. 1993. Sequential chromosome banding and in situ hybridization analysis. Genome, **36**: 792–795.

Different combinations of chromosome N- or C-banding with in situ hybridization (ISH) or genomic in situ hybridization (GISH) were sequentially performed on metaphase chromosomes of wheat. A modified N-banding–ISH/GISH sequential procedure gave best results. Similarly, a modified C-banding – ISH/GISH procedure also gave satisfactory results. The variation of the hot acid treatment in the standard chromosome N- or C-banding procedures was the major factor affecting the resolution of the subsequent ISH and GISH. By the sequential chromosome banding – ISH/GISH analysis, multicopy DNA sequences and the breakpoints of wheat–alien translocations were directly allocated to specific chromosomes of wheat. The sequential chromosome banding– ISH/GISH technique should be widely applicable in genome mapping, especially in cytogenetic and molecular mapping of heterochromatic and euchromatic regions of plant and animal chromosomes.

Key words: N-banding, C-banding, in situ hybridization, genomic in situ hybridization.

JIANG, J., et GILL, B.S. 1993. Sequential chromosome banding and in situ hybridization analysis. Genome, **36** : 792–795.

Différentes combinaisons de révélation des bandes C ou N par hybridation in situ (ISH) des chromosomes ou par hybridation in situ des génomes (GISH) ont été expérimentées de façon sériée sur des chromosomes de blé en métaphase. Une méthode sériée modifiée de révélation des bandes N par ISH/GISH a donné les meilleurs résultats. De façon similaire, une méthode sériée de révélation des bandes C par ISH/GISH a également donné des résultats satisfaisants. La variation du traitement à l'acide chaud dans les méthodes standards de révélation des bandes C et N des chromosomes a été le facteur majeur qui a affecté la résolution des traitements ISH/GISH subséquents. Par analyses des ISH/GISH révélant de façon sériée les bandes chromosomiques, les séquences d'ADN en multicopies et les points de fracture « blé-translocation étrangère » ont été directement assignés à des chromosomes spécifiques du blé. La technique de révélation sériée ISH/GISH des bandes chromosomiques devrait être largement applicable pour la cartographie des génomes et, plus spécifiquement, la cartographie cytogénétique et moléculaire des régions hétérochromatiques et euchromatiques des chromosomes des plantes et des animaux.

Mots clés : bandes N, bandes C, hybridation in situ, hybridation in situ des génomes.

[Traduit par la rédaction]

Introduction

Chromosome banding and in situ hybridization are two pivotal techniques for cytogenetics. Identification of individual chromosomes of human and most of the animal and plant species was impossible until the development of various chromosome banding techniques (for review see Hsu 1973). In situ hybridization is not only the most direct method for physical mapping of DNA sequences on chromosomes but also an alternative method for chromosome identification with different DNA markers.

In humans, chromosome banding and in situ hybridization techniques were successfully integrated into a single procedure. DNA sequences can be directly and more precisely mapped to a specific chromosome area by combining these techniques (see review by McNeil et al. 1991). Although both chromosome banding and in situ hybridization have been successfully applied for cytogenetic studies in plants, especially in wheat (*Triticum aestivum*) and its related species, reports on the sequential use of the two techniques have been limited (Hutchinson and Seal 1983). Thus, the potential of developing sequential banding – in situ hybridization techniques for plant chromosome analysis has not been fully exploited. Here, we report on the results of sequential analysis on wheat metaphase chromosomes using different combinations of N- or C-banding with in situ hybridization (ISH) or genomic in situ hybridization (GISH).

Materials and methods

Wheat variety 'Chinese Spring' (CS) and a wheat germplasm line KS92WGRC19 containing wheat–rye (*Secale cereale*) translocations T1AL·1RS and T4BS·4BL–6RL (Friebe et al. 1991) were used as the test materials.

The chromosome preparation and N-banding technique were according to Endo and Gill (1984). To integrate the N-banding technique with in situ hybridization, the major

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modification was the omission of the hot 45% acetic acid treatment. After removing the cover slips, the slides were dried in an ethanol series (75, 95, and 100% ethanol, 5 min each), then directly incubated in hot phosphate buffer (1 M NaH_2PO_4) at 92 ± 2°C for 2 min, rinsed with tap water, and immediately stained with a Banco Giemsa solution (dilute one drop of stock solution with 1 mL of 1/15 M Sørenson's phosphate buffer) for 22–24 min. After recording cells with well-spread N-banded metaphase chromosomes on film, the slides were destained in an ethanol series (75, 95, and 100% ethanol, 5 min each) and were processed for in situ hybridization. The same cells were rephotographed after in situ hybridization and the results were compared.

The C-banding technique was as described (Gill and Kimber 1974; for review see Gill et al. 1991). The modification of the C-banding procedure for sequential C-banding – in situ hybridization analysis included varying the duration and temperature of the 0.2 M HCl treatment. In addition to the standard treatment of chromosome preparations in 0.2 M HCl at 60°C for 2 min, treatment at 37°C and room temperature for 10–40 min were applied. After taking photos of the well-spread C-banded metaphase chromosome figures, the slides were destained in an ethanol series and were processed for in situ hybridization. The same metaphase chromosome figures were rephotographed after in situ hybridization and the results were compared.

ISH and diaminobenzidine tetrahydrochloride (DAB) detection techniques were according to Rayburn and Gill (1985). GISH and fluorescence detection techniques were as described (Le et al. 1989; Mukai and Gill 1991; Friebe et al. 1993). A ribosomal gene clone was used as a probe in ISH. This probe contains a single wheat 18S·26S rRNA gene repeat unit that originated from plasmid pTa71 (Gerlach and Bedbrook 1979).

Results

We tried both ISH/GISH – N-banding and N-/C-banding – ISH/GISH sequential combinations. The results of the sequential ISH/GISH – N-banding experiments showed that (*i*) the quality of N-banding following ISH with different probes was always poor; (*ii*) it was difficult to remove the hybridization signal detected with the DAB method (the banding result was better after fluorescence than after DAB detection); and (*iii*) it was impossible to get good banding following GISH.

The experiments involving sequential N-/C-banding – ISH/GISH procedures were much more successful. Despite some loss of resolution, both ISH and GISH were successfully performed after the standard N-banding. The resolution was improved by deleting the hot acetic acid treatment used in the standard N-banding procedure. Although the quality of the bands using the modified N-banding technique was usually not as good as that of the standard technique, all the N-bands on 16 pairs of CS wheat were consistently observed (Endo and Gill 1984). The smallest 18S·26S ribosomal gene cluster on chromosome 7D of CS (see Mukai et al. 1991) was localized with the modified N-banding – ISH procedure, indicating little loss of the ISH resolution (Fig. 1).

Figures 2*a* and 2*b* show a part of a cell of KS92WGRC19 by the procedure of modified N-banding – GISH using genomic DNA of rye as a probe. T1AL·1RS had no N-bands. T4BS·4BL–6RL showed a large N-band near the centromere on both arms. The breakpoints on both translocation chromosomes were clearly localized by the subsequent GISH.

Standard C-banding – ISH/GISH combinations reduced

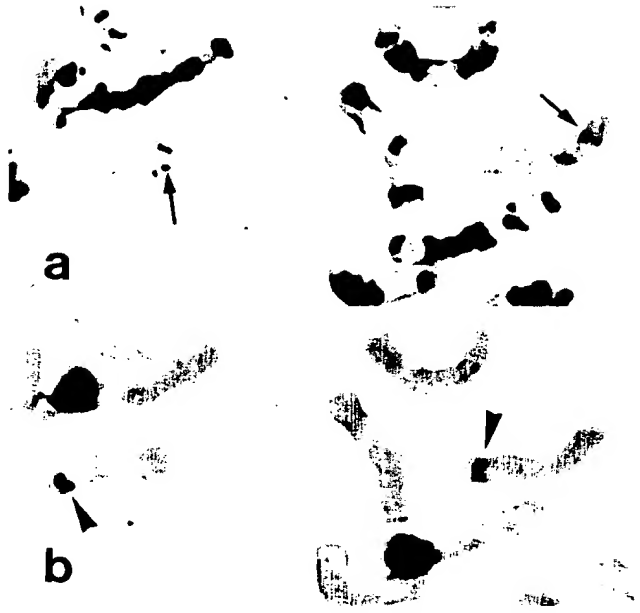

FIG. 1. Sequential modified N-banding – ISH (using 18S·26S ribosomal genes as a probe) analysis of 'Chinese Spring' (CS) wheat. (*a*) A part of a cell of CS with the modified N-banding. (*b*) The same cell after ISH. The N-band (arrow) on the short arm is diagnostic for the identification of this chromosome as 7D. The subsequent ISH locates an rRNA locus on the long arm of 7D (arrowhead).

the resolution of the ISH/GISH compared with N-banding – ISH/GISH combinations. The resolution of ISH and GISH was improved by the use of a modified C-banding procedure. In one experiment, we found that if the chromosome preparations were incubated in 0.2 M HCl at room temperature for 30 min, then the resolution of the GISH was better than with the standard HCl treatment. Most of the C-bands were visualized, although the quality of C-banding was decreased owing to the modification of the HCl treatment.

A part of a cell of KS92WGRC19 with modified C-banding – GISH procedure is shown in Figs. 2*c* and 2*d*. The rye-specific C-bands on 1RS of T1AL·1RS are clearly visible. Faint rye-specific C-bands on 6RL are also visible on T4BS·4BL–6RL. Although the contrast between the rye chromosome arm/segment and the wheat chromosomes in the subsequent GISH was not as good as that with the modified N-banding – GISH method, the location of breakpoints of the wheat–rye translocation chromosomes are still clear.

Discussion

Various combinations of chromosome banding techniques, i.e., G-, Q-, and replication banding, with in situ hybridization are routinely used in human chromosome analysis (see review by McNeil et al. 1991). Most of the combined techniques need separate photographic processes for the banding and in situ hybridization analyses. However, the techniques of simultaneous visualization of ISH signals and G-bands identified by bromodexyuridine (BrdU) incorporation are also available (Bhatt et al. 1988; Lawrence et al. 1990). These techniques have greatly facilitated

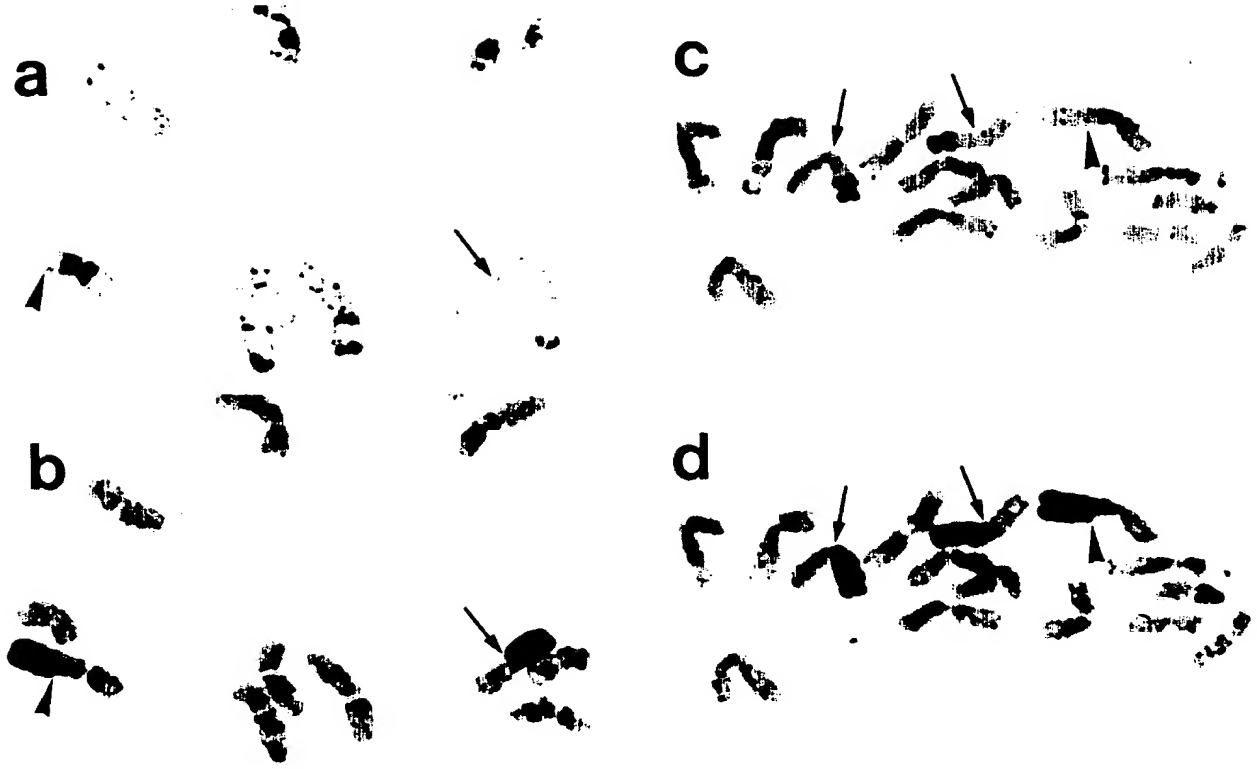

FIG. 2. Sequential modified N-/C-banding – GISH analysis of a wheat–rye translocation line KS92WGRC19. Arrows and arrowheads point to the breakpoints of the wheat–rye translocation chromosomes. Arrows, translocation chromosomes T1AL·1RS; arrowheads, translocation chromosomes T4BS·4BL–6RL. (*a*) A part of a cell of KS92WGRC19 with modified N-banding. (*b*) The same cell after GISH. (*c*) A part of a cell with modified C-banding (0.2 M HCl at room temperature for 30 min). (*d*) The same cell after GISH.

the physical mapping of human genes by ISH, as they allow chromosome identification and gene localization in one step.

The quality of banding after ISH may be variable and less optimal owing to morphological changes during chromosome denaturation (Lichter et al. 1991). The Giemsa–trypsin technique produces the highest resolution banding pattern for human chromosomes. However, trypsinization weakens the ISH signal (Lawrence et al. 1990). Thus, the application of this technique was limited. Replication banding and some other banding techniques, such as DAPI staining, do not interfere with ISH and are more widely used (McNeil et al. 1991).

In plants, sequential ISH (using radioactive probe) – C-banding analysis was reported on rye chromosomes (Hutchinson and Seal 1983). The resolution of the subsequent C-banding was reduced according to the authors' illustration. Sequential ISH (using biotin-labeled probes) – N-banding technique gave poor results in our hands. Furthermore, the results of GISH – N-banding were even more unsatisfactory.

Based on our results, the modified N-banding – ISH (using 18S·26S ribosomal genes as a probe) and modified N-banding – GISH gave excellent resolution. In fact, we consistently obtain better ISH and GISH contrast using the above procedures compared with standard ISH or GISH (without prior banding) using the same probes and same materials. It is not known why the contrast of ISH and GISH was improved after the modified N-banding. The hot acetic acid and HCl treatments in the regular N- and C-banding procedures are probably the major factors affecting the resolution of the subsequent ISH and GISH.

Although the modified N-banding – ISH/GISH gave the best results in the present study, unfortunately, not all the wheat chromosomes are marked with N-bands. In fact, the N-banding technique has been applied only to a limited number of plant species for chromosome identification, whereas C-banding is the most widely applicable banding technique in plants (Gill and Sears 1988). Therefore, we tried several modifications of the C-banding – ISH/GISH procedures. By modification of the 0.2 M HCl treatment in the present case, the modified C-banding–GISH technique showed sufficient resolution to locate the breakpoints and specific chromosomes in wheat–rye translocations. Therefore, the sequential C-banding – ISH technique should find wide application in cytogenetic and molecular analysis of chromosome structure and function.

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Bhatt, B., Burns, J., Flannery, D., and McGee, O'D. 1988. Direct visualization of single copy genes on banded meta-

phase chromosomes by nonisotopic *in situ* hybridization. Nucleic Acids Res. **16**: 3951–3961.

Endo, T.R., and Gill, B.S. 1984. Somatic karyotype, heterochromatin distribution, and nature of chromosome differentiation in common wheat, *Triticum aestivum* L. em Thell. Chromosoma, **89**: 361–369.

Friebe, B., Hatchett, J.H., Gill, B.S., Mukai, Y., and Sebesta, E.E. 1991. Transfer of Hessian fly resistance from rye to wheat via radiation-induced terminal and intercalary chromosomal translocations. Theor. Appl. Genet. **83**: 33–40.

Friebe, G., Jiang, J., Gill, B.S., and Dyck, P.L. 1993. Radiation-induced nonhomoeologous wheat – *Agropyron intermedium* chromosomal translocations conferring resistance to leaf rust. Theor. Appl. Genet. **86**: 141–149.

Gerlach, W.L., and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. **7**: 1869–1885.

Gill, B.S., and Kimber, G. 1974. Giemsa C-banding and the evolution of wheat. Proc. Natl. Acad. Sci. U.S.A. **71**: 4086–4090.

Gill, B.S., and Sears, R.G. 1988. The current status of chromosome analysis in wheat. *In* Chromosome structure and function. *Edited by* J.P. Gustafson and R. Appels. Stadler Genet. Symp. **18**: 299–321.

Gill, B.S., Friebe, B., and Endo, T.R. 1991. Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). Genome, **34**: 830–839.

Hsu, T.C. 1973. Longitudinal differentiation of chromosomes. Annu. Rev. Genet. **7**: 153–176.

Hutchinson, J., and Seal, A.G. 1983. A sequential *in situ* hybridization and C-banding technique. Heredity, **51**: 507–509.

Lawrence, J.B., Singer, R.H., and McNeil, J.A. 1990. Interphase and metaphase resolution of different distances within the human dystrophin gene. Science, (Washington, D.C.), **249**: 928–932.

Le, H.T., Armstrong, K.C., and Miki, B. 1989. Detection of rye DNA in wheat–rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Mol. Biol. Rep. **7**: 150–158.

Lichter, P., Boyle, A.L., Cremer, T., and Ward, D.C. 1991. Analysis of genes and chromosomes by nonisotopic *in situ* hybridization. Genet. Anal. Tech. Appl. **8**: 24–35.

McNeil, J.A., Johnson, C.V., Carter, K.C., Singer, R.H., and Lawrence, J.B. 1991. Localizing DNA and RNA within nuclei and chromosomes by fluorescence *in situ* hybridization. Genet. Anal. Tech. Appl. **8**: 41–58.

Mukai, Y., and Gill, B.S. 1991. Detection of barley chromatin added to wheat by genomic *in situ* hybridization. Genome, **34**: 448–452.

Mukai, Y., Endo, T.R., and Gill, B.S. 1991. Physical mapping of the 18S·26S rRNA multigene family in common wheat: identification of a new locus. Chromosoma, **100**: 71–78.

Rayburn, A.L., and Gill, B.S. 1985. Use of biotin-labeled probes to map specific DNA sequences on wheat chromosomes. J. Hered. **76**: 78–81.

Theor Appl Genet (1984) 67:463–467

Flow-cytometric characterization and sorting of plant chromosomes

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Summary. Flow cytometric measurements of DNA frequency distribution were used to follow the synchronization process in suspension cells from *Haplopappus gracilis* (2n = 4). Metaphase chromosomes were isolated from these synchronized cells and both the acro- and metacentric chromosomes were sorted by flow cytometry based on the different DNA contents. Possible applications of this procedure in fundamental genetics as well as practical plant breeding are discussed.

Key words: Chromosome isolation – Flow cytometry – *Haplopappus gracilis* (Nutt.) Gray – Synchronization

Introduction

Genetic manipulation may offer a valuable addition to conventional plant breeding methods. A main advantage is the potential to transfer parts of genomes containing only one or a few desirable genetic attributes to cells of a productive crop. Moreover, sexual incompatibility barriers are avoided. Transfer of genetic material may be accomplished in various ways. The transgenome may consist of a whole genome (cell hybridization), of intact chromosomes or parts of chromosomes (chromosome transplantation) or of DNA fragments in the kilobase range (monofactorial transformation).

Because of its high specificity, attention is mainly focussed on monofactorial transformation. However, the practical application of the method is largely limited by the lack of suitable procedures for identification of the gene(s) involved in most important plant characteristics. Moreover, the regulation of eukaryotic gene expression, especially in connection with cellular differentiation, is barely understood so far.

Chromosome transplantation may be a powerful technique in mapping of mono- and polygenic plant characteristics and in the identification of plant genes. In addition, chromosome-mediated gene transfer may serve as a transformation technique as such. The fate of a transferred chromosome is unknown but since aneuploidy occurs frequently in plants such chromosomes may be expected to behave autonomously.

Since the first isolation of mammalian chromosomes (Chorazy et al. 1963), considerable progress has been made. Metaphase chromosomes have been sorted by e.g. flow cytometry based on differences in DNA content or base constitution (Carrano et al. 1979). Chromosome-mediated gene transfer has also been achieved (Klobutcher and Ruddle 1981) and chromosome-specific gene libraries have been constructed (Davies et al. 1981).

Recently chromosomes have been isolated from several plant species (Malmberg and Griesbach 1980; Szabados et al. 1981; Griesbach et al. 1982; Hadlaczky et al. 1983).

Our study is focussed on the analysis of the synchronization process and on the purification and sorting of plant metaphase chromosomes by flow cytometry. Cell suspensions of *Haplopappus gracilis* were used as a model system since a) their synchronization has been well established (Eriksson 1966), b) the chromosome number is low (2n = 4) and c) the acro- and metacentric chromosomes are easy to distinguish morphologically and differ considerably in length (Jackson 1957).

Materials and methods

Plant material and growth conditions

Suspension cells of *Haplopappus gracilis* (Nutt.) Gray were initiated and maintained as described in detail by Werry and

Stoffelsen (1978). The synchronization procedure of Eriksson (1966) was adopted with some minor modifications. One day after subculturing, DNA synthesis was inhibited by 5 mM hydroxyurea (HU) for 18 or 24 h. The cells were subsequently washed thoroughly and exposed for 10 h to 0.05% colchicine for accumulation of mitotic cells. At various times during synchronization lactopropionic orcein-stained preparations were made (Dyer 1963) to determine the mitotic indices.

Frequency distributions of the DNA content of these cells were obtained by making protoplasts from log-phase, HU-treated and colchicine-treated cells respectively, according to Puite and Ten Broeke (1983). Cells were then incubated in 5% driselase (Kyowa Makko Kogyo Co, Tokyo, Japan) and 0.5% pectolyase (Seishia Pharmaceutical, Chiba-ken, Japan) in 0.7 M mannitol (pH 5.6) for 3 h at 28 °C in darkness. Protoplasts were washed and treated with 0.1% Triton X-100 and 50 μg ml^{-1} ethidium bromide (EtBr, a fluorescent DNA stain) in 0.5 M mannitol and used for flow cytometry directly.

Chromosome isolation

During the synchronization of the cell suspensions, the temperature was changed from 28 °C to 12 °C at 6 h after the addition of colchicine. Eight hours later, cells were exposed to 5% driselase, 0.5% pectolyase in 3 mM MES, 5 mM $MgCl_2$, 2 mM $CaCl_2$, 370 mM mannitol and 250 mM glucose (pH 5.6) (Hadlaczky et al. 1983) for 2 h. All following steps were carried out on ice. After washing and swelling (300 mM mannitol; 10 min) the nascent protoplasts were lysed in a solution containing 15 mM Hepes, 1 mM EDTA, 15 mM DTT, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 300 mM sucrose, 50 μg ml^{-1} (EtBr) and 1% Triton X-100 (pH 7.0) (Griesbach et al. 1982) and passed twice through a 25G hypodermic needle. Crude samples, thus obtained, were used for flow-cytometric analysis and sorting of the chromosomes.

Flow cytometry and sorting

Flow cytometric measurements and sorting experiments were performed with a FACS-IV Cell Sorter (Becton Dickinson, Sunnyvale, USA) equipped with a Spectra Physics argon ion laser, model 164-05, operated at 0.3 W/488 nm with a LP-620 filter in the emission beam (Donner et al. 1972). The fluorescence signal from the EtBr-stained preparations is considered to be proportional to the DNA content of the passing protoplasts, nuclei or chromosomes. The flow rate was approximately 1,000 particles per second. The DNA measurements gave rise to frequency distribution histograms with several peaks representing particles with the same DNA content. About 20,000 particles from each peak were sorted out into an ice-cooled tube containing 50% ethanol. Sorted fractions were kept on ice.

Flurescence microscopy

For identification of the sorted fractions the preparations were restained with EtBr (50 μg ml^{-1}), concentrated on 0.2 μm teflon millipore filters (Sartorius) and embedded in 50% glycerin. Fluorescence microscopy involved the use of a UG-2 exciter filter, a FL-500 dichroic mirror and a LP-580 emission filter.

Results and discussion

In order to isolate chromosomes from plant cells it is essential to dispose of large numbers of mitotic cells. Therefore, log-phase cells from *Haplopappus gracilis* were synchronized by incubation in HU and subsequently in colchicine (Eriksson 1966). In Fig. 1 the time course of the mitotic index (MI) during the synchronization of the suspension cells is demonstrated. During the HU treatment MI decreases from 4% in the log-phase control to less than 1%. From 6 h after washing onwards, MI rapidly increases. Mitotic indices of about 25% were routinely obtained (Fig. 2).

The synchronization process was also studied by flow cytometry by measuring the frequency distributions of the DNA content of burst protoplasts after various incubation periods (Fig. 3). In log-phase suspensions, cells were mainly in G_0/G_1 phase (approx. 80%) whereas only a few cells were found in S (5%) and G_2/M phase (15%) (Fig. 3 A). However, as expected, after incubation in hydroxy urea and colchicine, cells were accumulated in the S phase (Fig. 3 B) and G2/M phase (Fig. 3 C) respectively.

Since the cell wall is a main barrier for the isolation of plant chromosomes, protoplasts were prepared by treatment with cell wall-digesting enzymes in 0.7 M mannitol. However, during protoplast preparation from mitotic cells at 28 °C, we observed a rapid despiralization of the metaphase chromosomes. The chromosomes were largely protected from decondensation by changing the temperature from 28 °C to 12 °C and by using a special buffer solution (Hadlaczky et al. 1983) during a 2 h cell wall digestion. Although cell walls were not completely digested, they were sufficiently weakened to allow mechanical breakage in order to obtain the chromosomes.

The lysis buffer was composed as described by Griesbach et al. (1982) and completed with 50 μg ml^{-1} ethidium bromide (EtBr) and 1% Triton X-100. The intercalator EtBr acted not only as a fluorescent DNA-stain but also as a DNA stabilizing factor (J. Aten, personal communication). Moreover, EtBr can be

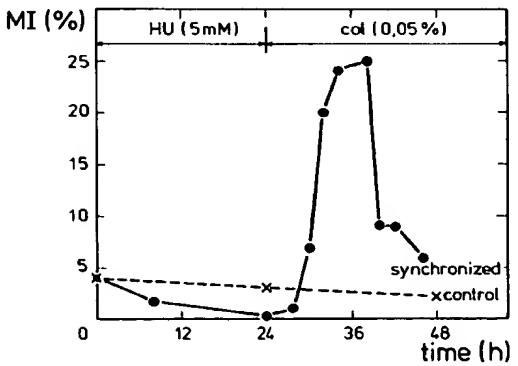

Fig. 1. Time course of the mitotic index (MI) of *Haplopappus gracilis* suspension cells during synchronization

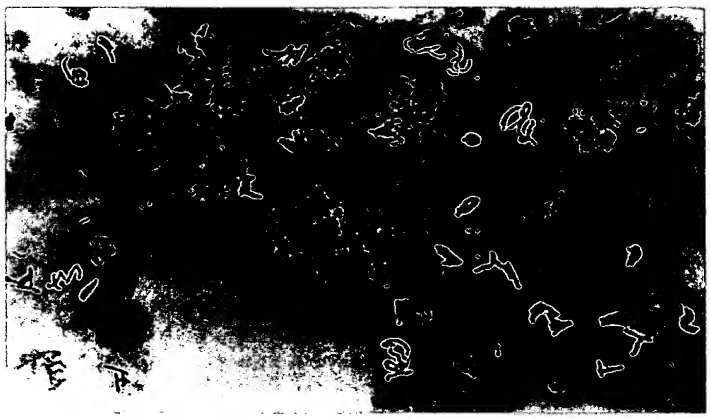

Fig. 2. Lactopropionic orcein-stained squash preparation of synchronized *Haplopappus* cells

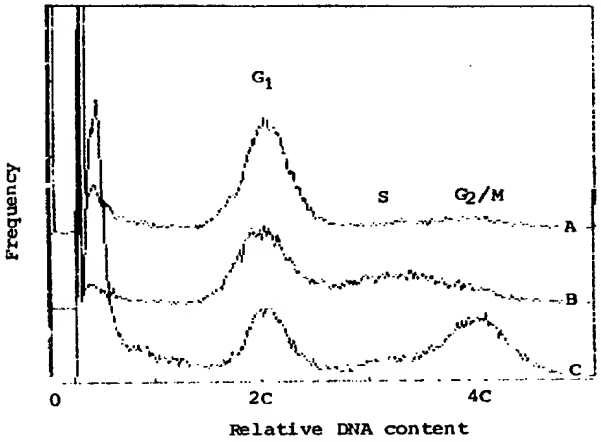

Fig. 3. Frequency distribution of the relative DNA content of protoplasts prepared from log-phase (*A*), HU-treated (*B*) and colchicine-treated (*C*) suspension cells

washed out, probably without affecting the biological activity of DNA. The presence of Triton X-100 (1%) greatly prevented the cell components from aggregation.

The flow cytometric analysis of crude protoplast extracts yielded 8 peaks (Fig. 4A–H) which were identified by flow sorting and fluorescence microscopy as debris (A) acrocentric chromosomes (B + C), metacentric chromosomes (D + E), G_1 nuclei (F), metaphase nuclei (aggregates of 4 chromosomes) (G) and G_2 nuclei (H). The fluorescence intensity of metaphase nuclei (G), is significantly lower than that of G_2 interphase nuclei (H) containing the same amount of DNA. This is probably a consequence of DNA condensation preventing EtBr to intercalate proportionally or leading to an increased quenching.

The coefficient of variations (CV) for the peaks containing chromosomes and nuclei routinely did not exceed the 4% and 9% level respectively.

Both types of chromosomes were sorted at a rate of 5×10^3 chromosomes min^{-1} (Fig. 5). Contamination of the acrocentric chromosome fraction (B + C) with metacentric chromosomes (D + E) or vice versa did not exceed the 5% level. Both the acrocentric and metacentric chromosome fractions consisted of double peaks, each containing equal amounts of chromosomes. The nature of these double peaks was investigated. For sperm cells, the effects of orientation during passage of the laser beam have already been described as resulting in double peaks (Gledhill et al. 1976). However, since

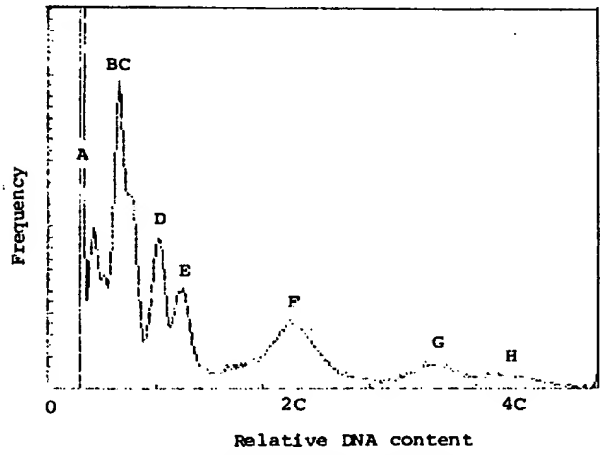

Fig. 4. Frequency distribution of the relative DNA content of EtBr-stained chromosomes and nuclei from mechanically disrupted protoplasts. By flow sorting, peaks were identified as debris (*A*), acrocentric (*B/C*) and metacentric (*D/E*) metaphase chromosomes, G_1 nuclei (*F*), mitotic nuclei (*G*) and G_2 nuclei (*H*)

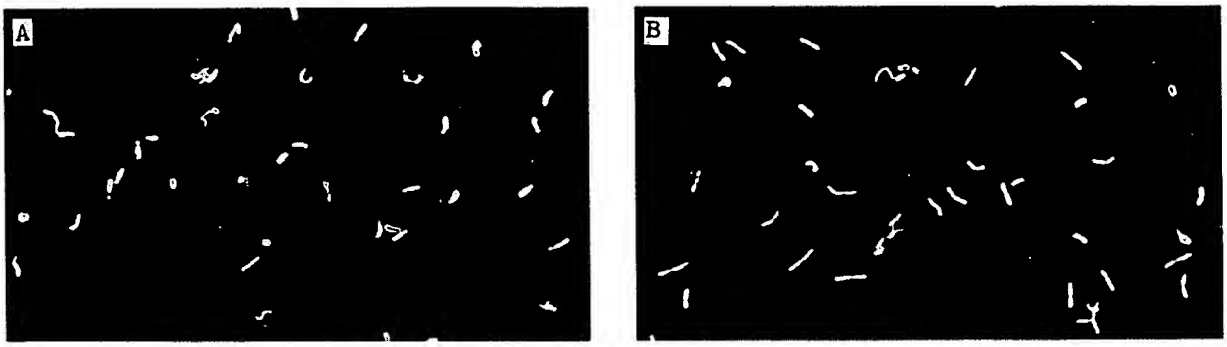

Fig. 5. Acrocentric (A) and metacentric (B) *Haplopappus* metaphase chromosomes as sorted by flow cytometry

the re-analysis of fraction D and E exhibited only one peak each, the involvement of such reversible chromosome orientation could be excluded. If the double peaks reflect differences in fluorescence between the homologous chromosomes, these differences may result either from differences in DNA base constitution or DNA content. The former, however, is most unlikely since the intercalator, EtBr, is not base specific. To ascertain whether differences in DNA content of both homologues are involved in causing the double peaks, chromosome lengths were measured in synchronized suspension cells. Surprisingly, significant differences were found between the two homologues, the relative mean chromosome-lengths being 100 and 120 for the acrocentric and 155 and 181 for the metacentric chromosomes. This is in good agreement with the relative DNA contents as measured by flow cytometry: 100 and 116 for the acrocentric and 155 and 187 for the metacentric chromosomes. Thus, the differences between homologous chromosomes already existed in our cell suspension and did not result from possible damage during the isolation procedure. Since our cell suspension has been subcultured weekly for at least five years, the observed chromosome dimorphism might have resulted from genetic instability under some selection pressure (D'Amato 1975).

Conclusions

The synchronization process of *Haplopappus* suspension cells, routinely resulting in mitotic indices of about 25%, could be followed easily by flow cytometry. Since DNA histograms can be measured very rapidly, flow cytometry herewith proves its potency in cell cycle analyses, but also in studying genetic instability (determination of ploidy level, aneuploidy and karyotype alterations).

Sorting of plant chromosomes has not been described before. Since the CV for the chromosome peaks is about 4%, the procedure described here enables us to sort chromosomes differing in DNA content by about 10% at a rather useful rate (about 5×10^3 chromosomes per min) apparently without damaging the chromosomes. This provides the opportunity for transplantation of particular chromosomes via fusion techniques (Szabados et al. 1981; Griesbach et al. 1982) or by microinjection (Steinbiss 1983). This would create an important tool for plant breeding as well as gene mapping. Furthermore, chromosome-specific gene libraries may be constructed as has already been done in mammalian cells (Davies et al. 1981).

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References

Carrano AV, Gray JW, Langlois RG, Burkhart-Schultz KJ, Van Dilla MA (1979) Measurement and purification of human chromosomes by flow cytometry and sorting. Proc Natl Acad Sci USA 76:1382–1384

Chorazy M, Bendich A, Borenfreund E, Hutchinson D (1963) Studies on the isolation of metaphase chromosomes. J Cell Biol 19:59–69

D'Amato F (1975) The problem of genetic stability in plant tissue and cell cultures. In: Frankel O, Hawkes JG (eds) Crop genetic resources for today and tomorrow. Cambridge University Press, Cambridge, pp 333–348

Davies KE, Young BD, Elles RG, Hill ME, Williamson R (1981) Cloning of a representative genomic library of the human X chromosome after sorting by flow cytometry. Nature 293:374–376

Donner WA, Huett HR, Sweet RG, Herzenberg LA (1972) Fluorescence activated cell sorting. Rev Sci Instrum 43: 404–409

Dyer AF (1963) The use of lacto-propionic orcein in rapid squash methods for chromosome preparations. Stain Technol 38:85–90

Eriksson T (1966) Partial synchronization of cell division in suspension cells of *Haplopappus gracilis*. Physiol Plant 19:900–910

Gledhill BL, Lake S, Steinmetz LL, Gray JW, Crawford JR, Dean PN, Van Dilla MA (1975) Flow microfluorometric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution. J Cell Physiol 87:367–376

Griesbach RJ, Malmberg RL, Carlson PS (1982) Uptake of isolated lilly chromosomes by tobacco protoplasts. J Hered 73:151–152

Griesbach RJ, Malmberg RL, Carlson PS (1982) An improved technique for the isolation of higher plant chromosomes. Plant Sci Lett 24:55–60

Hadlaczky G, Bisztray G, Praznovszky T, Dudits D (1983) Mass isolation for plant chromosome and nuclei. Planta 157:278–285

Jackson RC (1957) New low chromosome number for plants. Science 126:1115–1116

Klobutcher LA, Ruddle FH (1981) Chromosome-mediated gene transfer. Ann Rev Biochem 50:533–554

Malmberg RL, Griesbach RJ (1980) The isolation of mitotic and meiotic chromosomes from plant protoplasts. Plant Sci Lett 17:141–147

Puite KJ, Ten Broeke WRR (1983) DNA staining of fixed and non-fixed plant protoplasts for flow cytometry with Hoechst 33342. Plant Sci Lett (in press)

Steinbiss HH, Stabel P (1983) Protoplast derived tobacco cells can survive capillary microinjection of the fluorescent dye lucifer yellow. Protoplasma 116:223–229

Szabados L, Hadlaczky G, Dudits D (1981) Uptake of isolated plant chromosomes by plant protoplasts. Planta 151: 141–145

Werry PAThJ, Stoffelsen KM (1978) Conditions for a high plating efficiency of free cell suspensions of *Haplopappus gracilis* (Nutt.) Gray. Theor Appl Genet 51:161–167

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Sensitivity enhancement of fluorescence *in situ* hybridization on plant chromosomes

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An improved *in situ* hybridization procedure is presented, based on synchronization of root meristems of barley and wheat, enzymatic digestion, a protoplast drop technique, and the use of the fluorescent dye Cy3. The combination of these approaches resulted in a significant increase of well-spread metaphases suitable for *in situ* hybridization as compared to squash preparations, and to a significantly enhanced number and intensity of hybridization signals as demonstrated for a B-hordein-specific low-copy probe of barley. In the case of Cy3 all metaphases displayed a signal, more than 60% of them on both chromatids of each gene-bearing chromosome.

Key words: B-hordein, cell cycle synchronization, drop spread technique, fluorescence *in situ* hybridization

Introduction

In situ hybridization has become an increasingly powerful tool since its first description by Gall & Pardue (1969) and since then it has been possible to detect low-copy and even single-copy sequences on human chromosomes by fluorescence *in situ* hybridization (FISH) with remarkable resolution (e.g. Lawrence *et al.* 1988, Viegas-Pequignot *et al.* 1989, Lichter *et al.* 1990). The visualization of such sequences on plant chromosomes lags behind, however. The few examples available have employed a variety of techniques (Ambros *et al.* 1986, Mouras *et al.* 1987, Huang *et al.* 1988, Lehfer *et al.* 1991, 1993) but exclude the demonstration of single-copy sequences using FISH.

Apart from the fact that the cell wall can affect the accessibility of the probe to a chromosome, and that cellular debris often causes a relatively high background in the commonly used squash pre-parations, one of the principal problems of this pre-paration technique is the generally low number of suitable metaphases per slide. Ambros *et al.* (1986) have attempted to bypass this problem by synchronizing cell divisions in root meristems and by the use of protoplast drop techniques, but the autoradiographic approach chosen is inferior to fluorescence detection. It is time consuming and does not allow the exact localization of sequences. We have combined three recent experimental improvements, a synchronization method (Doležel *et al.* 1992), the protoplast drop technique and modified *in situ* hybridization procedures, and achieved an encouraging improvement in detection efficiency.

Materials and methods

Plant material and chromosome preparation

Seeds of barley (*Hordeum vulgare* L.) cv. Igri, the ditelotetrasomic barley line 1HS (14 + 2t) and the wheat-barley addition line 3HS (42 + 2t) were used in this study. Seeds were germinated on moist filter paper at 22°C in the dark.

Improvement of metaphase indices with the drop spread technique was compared with other methods. Roots of young seedlings were cut off and stored in icewater overnight or incubated in 0.05% colchicine for 4 h at room temperature. Alternatively, after incubation of young seedlings with hydroxyurea (HU) and APM (O-methyl-O-(2-nitro-*p*-tolyl)N-isopropylphosphoramidothioate, Amiprophosmethyl; Bayer, Leverkusen) roots were cut off and stored in icewater overnight as described in Doležel *et al.* (1992) & Pan *et al.* (1993). The material was then fixed in 3 : 1 (v/v) ethanol : acetic acid and stored at −20°C until use.

For chromosome preparations, roots were briefly rinsed in tap water, their tips (2–3 mm) cut off for digestion in 250 µl of 2.5% Pectolyase Y-23 (Kikkoman, Düsseldorf), 2.5% Cellulase 'Onozuka'

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R-10 (Serva, Heidelberg) in 75 mM KCl and 7.5 mM EDTA (pH 4.0) at room temperature for 50 min as detailed in Pan *et al.* (1993). The lysate of 5–15 root tips was filtered through a 55 µm mesh net, the protoplasts were suspended in a total volume of 10 ml with 75 mM KCl and spun down after 5 min at 80 × *g* for 5 min. The pellet was gently resuspended in 3 : 1 fixative and the cells were spun down again. The last step was repeated four times. Cells were finally resuspended in an appropriate volume of fixative (100–250 µl) and dropped on icecold, cleaned slides.

Probes and probe preparation

Three different probes were used: first, the rDNA-specific probe CHS50 which carries 700 bp of the barley 25S rDNA (data not shown), second a 220 bp repetitive probe, designated MWG1007, isolated by microdissection and microcloning of the barley chromosome 1HS (Schondelmaier *et al.* 1993). This probe contains an AT-rich segment of 98 bp and displays no similarity to known sequences. The third probe, the low-copy DNA fragment pBSC21 (Siedler & Graner 1991) is a 3.5 kb derivative of the *Hor*2-specific probe pchor 2-4 (Brandt *et al.* 1985). It originates in the distal part of the barley chromosome 1HS (Lehfer *et al.* 1991, 1993). MWG1007 and pBSC21 were labelled by nick translation (BioNick Markerkit, BRL, Eggenstein) with Biotin-14-dATP, and CHS50 by nick translation (nick translation kit, BRL) with Digoxigenin-11-dUTP (Boehringer, Mannheim). They were purified by a Sephadex G-50 spin column to remove unincorporated nucleotides, and by an ethanol precipitation step. For hybridization, MWG1007-Bio-14-dATP (final concentration 5 ng/µl) was dissolved in 60% deionized formamide, 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate), 1 mg/ml herring sperm DNA and 1 mg/ml *E. coli* tRNA. PBSC21-Bio-14-dATP (final concentration 10 or 20 ng/µl) was dissolved in 50% deionized formamide, 2 × SSC, 1 mg/ml herring sperm DNA, 1 mg/ml *E. coli* tRNA and 10% dextran sulphate. The same hybridization mix was used for double hybridization with pBSC21-Bio-14-dATP (5 ng/µl) and CHS50-Dig-11-dUTP (1.5 ng/µl).

In situ hybridization and signal detection

The slides were immersed in 3 : 1-fixative for 30 min at room temperature, dried and incubated with 100 µg/ml RNAse A (Sigma, München) for 60 min at 37°C in a moist chamber. After removal of coverslips, they were washed three times in 2 × SSC, pH 7.2, at room temperature for 5 min each and subsequently digested for 10 min in 0.005% Pepsin (Serva Heidelberg no 31855) in 0.01 M HCl at 37°C according to Wiegant *et al.* (1991). They were washed twice in 2 × SSC, pH 7.2, for 5 min and then dehydrated in a graded ethanol series (70%, 80%, 90%, 96%, 2 min each). Twelve µl of DNA probe were loaded per slide, sealed with a coverslip and rubber cement, and the samples were denatured in a waterbath at 70°C for 2 min. After hybridization in a moist chamber at 37°C overnight, the coverslips were removed by washing in 2 × SSC, pH 7.2 (room temperature). The samples were then washed in 50% formamide, 2 × SSC, pH 7.2, at 45°C (60% formamide, 2 × SSC, pH 7.2, for the repetitive probe at room temperature); 2 × SSC, pH 7.2, three times 5 min each; once in 0.05% Tween-20, 4 × SSC, pH 7.2, at room temperature, followed by a blocking step as described by Wiegant *et al.* (1991), using 0.5% blocking agent, BA, (Boehringer, Mannheim) and 4 × SSC, pH 7.2, at 37°C for 30 min).

Signal detection of biotinylated probes was performed according to Pinkel *et al.* (1988). After incubation (37°C, 30 min) with avidin-FITC (5.4 µg/ml; Sigma, München) or avidin-Cy3 (1 µg/ml or 0.4 µg/ml; Dianova, Hamburg) both dissolved in 0.5% BA, 4 × SSC, pH 7.2, slides were washed three times in 0.05% Tween-20, 4 × SSC, pH 7.2, at 45°C for 5 min. They were then incubated with goat anti-avidin D antibody (1.25 µg/ml, 37°C, 30 min; Vector Labs., Burlingame, USA) and dissolved in 0.5% BA, 4 × SSC, pH 7.2.

After washing as described above, a second layer of avidin-FITC was applied. An additional signal amplification cycle was included for the detection of the low-copy probe pBSC21.

For double labelling, additional antibodies were added to the detection mixture to detect the digoxigenin-labelled probe. Mouse anti-digoxigenin antibody (0.2 µg/ml; Boehringer) at the first step, TRITC (tetramethylrhodamine isothyocyanate)-conjugated rabbit anti-mouse antibody (1 : 500; Sigma) at the second step, and TRITC-conjugated goat anti-rabbit antibody (1 : 500; Sigma) at the third step.

After the final washing in 0.05% Tween-20, 4 × SSC, pH 7.2, slides were rinsed twice in phosphate-buffered-saline (PBS: 0.1 M phosphate, 0.13 M NaCl, pH 7.2) at room temperature and dehydrated in a graded ethanol series as described above. Chromosomes were counterstained with propidium iodide or diamidinophenylindole (DAPI), 0.5 µg/ml each, in antifade solution (Johnson & Arujo Nogueira 1981).

Microscopy

Labelling efficiency was determined by counting the hybridization signals of metaphases and interphases using a Zeiss Axioplan epifluorescence microscope equipped with filter sets 487901 (DAPI), 487909 (FITC, Cy3) and 487915 (TRITC, Cy3). Photographs

were taken with a Zeiss MC100 camera and a Fuji 400D professional slide film.

Results

During a test series of different synchronization methods for barley in combination with the drop technique, the procedure of Doležel *et al.* (1992) as modified by Pan *et al.* (1993) provided the highest number of well-spread metaphases (Figure 1). This approach also proved useful for wheat.

Figure 2(C) illustrates a metaphase of barley after hybridization with the barley-specific repetitive probe MWG1007. All 14 barley chromosomes are labelled uniformly, with the exception of their centromeric region. In the wheat–barley addition line the two added barley chromosomes (3HS) can be identified both by their selective staining (Figure 2A) and shape (Figure 2B). The drop technique and digestion with pepsin reduced cytoplasma and hence background significantly. This is also evident in double labelling experiments, illustrated by Figure 2D.

The *Hor2* locus, recently mapped to the distal end of the short arm of barley chromosome 1H (Lehfer *et al.* 1991, 1993), was chosen to monitor the labelling efficiency for a low-copy sequence.The *Hor2*-specific signals of probe pBSC21 per metaphase, counted for both fluorescence dyes, gave a labelling efficiency for the four chromatids of both homologous chromosomes of 59% for FITC and 66% for Cy3 (Figure 3). In the case of Cy3 and a probe concentration of 10 ng/µl, no metaphase was without a specific signal. There was no notable difference in the labelling efficiency between both dyes with 10 or 20 ng of probe/µl hybridization mixture, except that the number of metaphases lacking a signal increased with the higher probe concentrations (data not shown), probably due to self-reassociation of single-stranded probe DNA in solution.

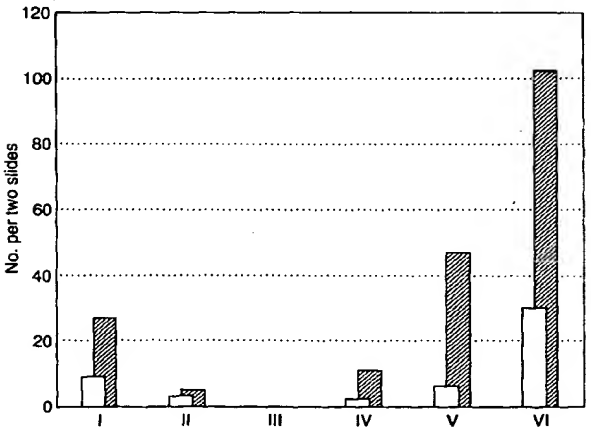


Figure 1. Quality of barley metaphase spreads for different synchronization methods ($2n = 14$): □, maximally two chromosomes are overlapping; ▨, 6–10 free chromosomes. Treatment was with: icewater (I), amiprophosphomethyl (APM; II), colchicine (III), hydroxyurea (HU)–icewater (IV), HU–APM (V) or HU–APM–icewater.

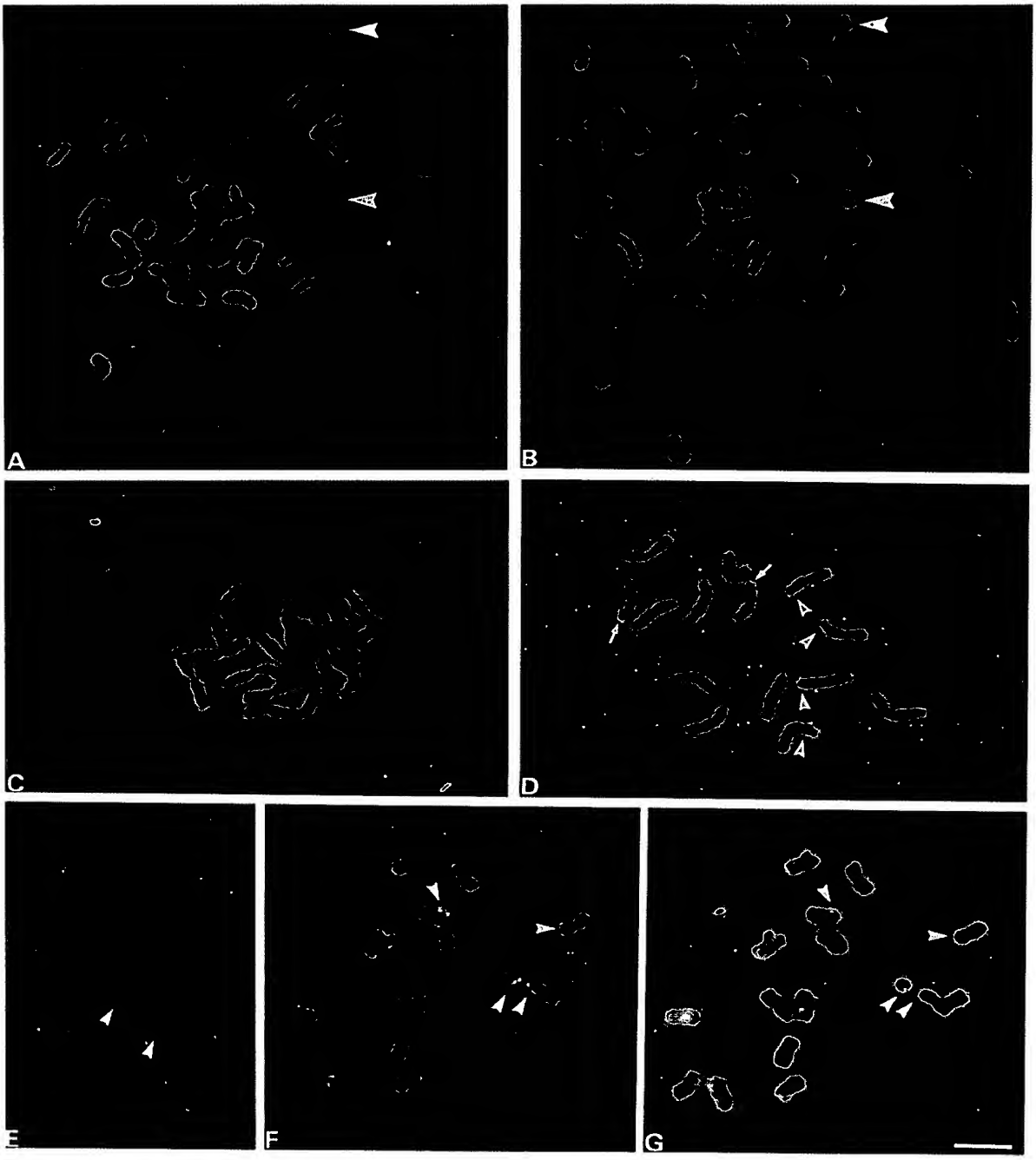
A substantial improvement in the detection of pBSC21 in interphase cells was obtained by Cy3. The use of FITC frequently resulted in relatively weak signals that could not be readily distinguished from background. On the other hand, Cy3 showed bright signals in interphases (Figure 2E) and could be excited not only by illumination with 546 nm (filter 487915), but also with 450–490 nm (filter 487909). Although the green signal observed in the latter instance was fainter than the usual red one, its advantage was that background signals were generally diminished more than the probe signal (Figure 2F, G), and no double exposure was necessary.

The number of interphase signals using either the Cy3 or FITC detection system is compared in Figure 4. Twenty-eight percent (FITC) and 25% (Cy3) of interphases carried a twin signal whereas only a small percentage of interphases was noted with two double signals (3.5% with FITC, 6.5% with Cy3). It should be mentioned that in 25% of all interphases a distinction between background or specific labelling was not possible.

Discussion

The outlined modifications of the *in situ* hybridization protocol improve the detection sensitivity of FISH for plant chromosomes substantially. They include (i) the increase of metaphase indices due to synchronization of plant material with hydroxyurea and the spindle toxin APM compared with that by treatment with colchicine or icewater (Figure 1), (ii) the drop technique which results in an increased number of well-spread and complete metaphases compared with standard squash preparations, (iii) the significant reduction of cytoplasmic background by digestion with pepsin instead of proteinase K, and (iv) the improvement of detection sensitivity by the use of the fluorescence dye Cy3 instead of fluorescein isothyocyanate (FITC) which results in brighter signals, and has a greater resistance to fading than FITC.

The *Hor2*-specific probe pBSC21, known to originate near the telomere of chromosome 1HS (Lehfer *et al.* 1991, 1993), has been chosen to establish the labelling efficiency statistically (Figures 2 & 3). The *Hor2* locus consists of approximately 12 genes which are arranged in two adjacent clusters, separated by a DNA segment of unknown length (Sørensen 1989). The sensitivity of signal detection in metaphases in comparison to customarily used squash techniques for this locus was increased significantly, especially for the frequency of signals on both chromatids of a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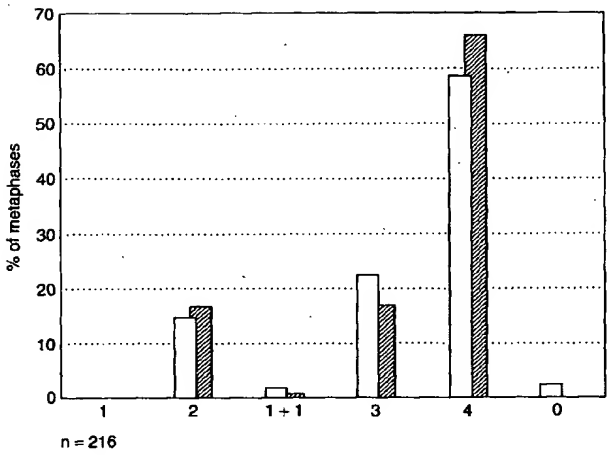

Figure 3. The number of marked chromatids per metaphase (in %); (1 + 1) = one signal per chromosome. The probe pBSC21 was dissolved to a final concentration of 10 ng/µl; av-FITC (□) was dissolved at a dilution of 5.4 µg/ml and av-Cy3 (▨) was dissolved at a dilution of 1 µg/ml.

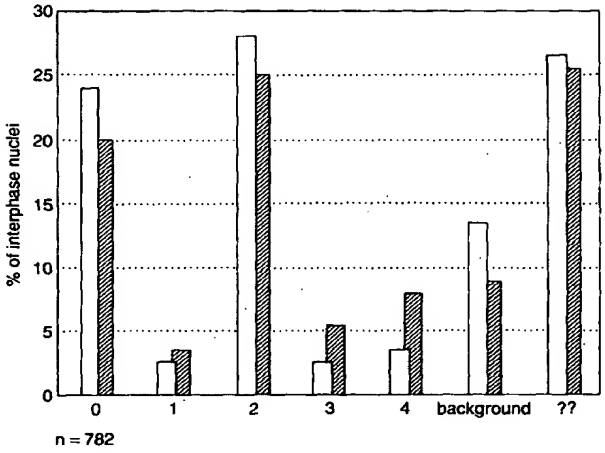

Figure 4. The number of signals per interphase nucleus (in %): av-FITC (□) was dissolved at a dilution of 5.4 µg/ml and av-Cy3 (▨) was dissolved at a dilution of 1 µg/ml. ?? = percentage of interphase nuclei, in which background and specific signals could not be distinguished.

chromosome. This is a crucial criterion for selecting chromosomes for detailed mapping (Lichter *et al.* 1990). The significantly lower frequency of signals and the higher background in interphases than in metaphases could be due to the preparation technique which was optimized for metaphase chromosomes (Figures 2 & 3).

Until now only highly repetitive probes have been used for simultaneous fluorescence visualization of different probes in cytological preparations of plant chromosomes (Lehfer *et al.* 1991, Leitch *et al.* 1991). The efficient demonstration of low-copy probes *per se* and in double labelling experiments (Figure 2D) can be advantageous for simultaneous probe mapping on plant chromosomes. In this work, TRITC was used for double labelling experiments. This fluorescence dye gives fainter signals than Cy3 (Wessendorf & Brelje 1992) and should be avoided in multiple labelling experiments if Cy3 can be used. The brighter Cy3 signals enhance the sensitivity for detection of sequences in interphase nuclei compared to FITC (Figure 3) so that it is the dye of choice in experiments when highest detection sensitivity is desired.

The improvement of non-radioactive *in situ* hybridization outlined in this study encourages an augmented application of FISH in plant research. Apart from the fact that it offers the opportunity to detect single-copy sequences in plants, the improved detection sensitivity could be an enormous advance for establishing topographical maps, including chromosomes, such as the barley chromosomes 2H, 3H, 4H and 7H which lack pronounced morphological markers for their unequivocal identification and hence cannot be identified by morphology. The use of genetically mapped chromosome-specific (restriction fragment length polymorphism) probes (Graner *et al.* 1990) can bypass this drawback and should allow exact localization of probes in metaphase chromosomes as well as their comparison with genetic distances.

Figure 2. (*Opposite*) **A** A metaphase spread of wheat–barley addition line 3HS after *in situ* hybridization with MWG1007 and probe visualization by Cy3. Only the barley chromosomes exhibit the hybridization signal. The somewhat discordant position of the Cy3 signal with the counterstaining is caused by the filter change required for taking the photographs for both dyes. **B** DAPI counterstaining signal of the metaphase shown in **A**. **C** A barley metaphase spread hybridized with the same probe as in **A**. The chromosomes are uniformly stained except in their centromeres. **D** Double-labelling of a barley metaphase spread with probe CHS50 and pBSC21. CHS50 was visualized by TRITC; pBSC21 by FITC. CHS50 hybridizes to the NOR chromosomes 5H and 6H (arrowheads). The double-band effect is caused by a 'bleed through' of the signal with a filter not specific for the wavelength of TRITC emission. pBSC21 is located at the distal end of chromosome 1HS (arrows). **E** Localization of pBSC21 in a late interphase of barley. Two hybridization sites can be seen, but only one is resolved into two spots representing the two chromatids of chromosome 1HS. **F & G** In the barley ditelotetrasomic line 1HS, the pBSC21 signal can clearly be detected on both telosomes and on one chromosome 1H, whereas the second chromosome carries weak hybridization signals: **G** (double exposure) shows a higher background signal (see results and discussion) compared with **F** (single exposure), taken with filter combination 487909. Scale bar: 10 µm.

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References

Ambros PF, Matzke MA, Matzke AJM (1986) Detection of a 17 kb unique sequence (T-DNA) in plant chromosomes by *in situ* hybridization. *Chromosoma* **94**: 11–18.

Brandt A, Montembault A, Cameron-Mills V, Rasmussen SK (1985) Primary structure of a B1 hordein gene from barley. *Carlsberg Res Commun* **50**: 333–345.

Doležel J, Cihalikova J, Lucretti S (1992) A high-yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba* L. *Planta* **188**: 93–98.

Gall JG, Pardue ML (1969) Formation and detection of RNA–DNA hybrid molecules in cytological preparations. *Proc Natl Acad Sci USA* **63**: 378–383.

Graner A, Siedler H, Jahoor A, Herrmann RG, Wenzel G (1990) Assessment of the degree and type of restriction fragment length polymorphism in barley (*Hordeum vulgare*). *Theor Appl Genet* **80**: 826–832.

Huang PL, Hahlbrock K, Somssich I (1988) Detection of a single copy gene on plant chromosomes by *in situ* hybridization. *Mol Gen Genet* **211**: 143–147.

Johnson GD, Arujo Nogueira GM (1981) A simple method of reducing the fading of immunofluorescence during microscopy. *J Immunol Methods* **43**: 349–350.

Lawrence JB, Villnave CA, Singer RH (1988) Sensitive, high resolution chromatin and chromosome mapping *in situ*: presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell* **52**: 51–61.

Lehfer H, Wanner G, Herrmann RG (1991) Physical mapping of DNA sequences on plant chromosomes by light microscopy and high resolution scanning electron microscopy. In: Herrmann RG and Larkins B, eds. *Plant Molecular Biology 2*. New York: Plenum Press, pp. 277–284.

Lehfer H, Busch W, Martin R, Herrmann RG (1993) Localization of the B-hordein locus on barley chromosomes using fluorescence *in situ* hybridization. *Chromosoma* **102**: 428–432.

Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultaneous *in situ* hybridization of two differently labelled fluorescent probes. *Genome* **34**: 329–333.

Lichter P, Chang Tang C, Call K *et al.* (1990) High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* **24**: 64–69.

Mouras A, Saul MW, Essad S, Potrykus I (1987) Localization by *in situ* hybridization of a low copy chimaeric resistance gene introduced into plants by direct gene transfer. *Mol Gen Genet* **207**: 204–209.

Pan WH, Houben A, Schlegel R (1993) Highly effective cell synchronization in plant roots by hydroxyurea, and amiprophosmethyl or colchicine. *Genome* **36**: 387–390.

Pinkel D, Landegent J, Collins C *et al.* (1988) Fluorescence *in situ* hybridization with human chromosome specific libraries: Detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci USA* **85**: 9138–9142.

Schondelmaier J, Martin R, Jahoor A *et al.* (1993) Microdissection and microcloning of the barley (*Hordeum vulgare* L.) chromosome 1HS. *Theor Appl Genet* **86**: 629–636.

Siedler H, Graner A (1991) Construction of physical maps of the *Hor*1 locus of two barley cultivars by pulsed field gel electrophoresis. *Mol Gen Genet* **226**: 177–181.

Sørensen MB (1989) Mapping of the *Hor*2 locus in barley by pulsed field gel electrophoresis. *Carlsberg Res Commun* **54**: 109–120.

Viegas-Pequignot E, Dutrillaux B, Magdalent H, Coppey-Moisan M (1989) Mapping of single-copy DNA sequences on human chromosomes by *in situ* hybridization with biotinylated probes: Enhancement of detection sensitivity by intensified-fluorescence digital-imaging microscopy. *Proc Natl Acad Sci USA* **86**: 582–586.

Wessendorf MW, Brelje TC (1992) Which fluorophore is brightest? A comparison of the staining obtained using fluorescein, tetramethylrhodamine, lissamine rhodamine, Texas Red, and cyanine 3.18. *Histochemistry* **98**: 81–85.

Wiegant J, Galjart NJ, Raap AK, d'Azzo A (1991) The gene encoding human protective protein (PPGB) is on chromosome 20. *Genomics* **10**: 345–349.

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Differentiation of field bean heterochromatin by *in situ* hybridization with a repeated *Fok*I sequence

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The chromosomes of a field bean line with a reconstructed karyotype (ACB) were hybridized *in situ* with biotinylated probes of a repetitive *Fok*I sequence, of DOP-PCR (degenerate oligonucleotide primed polymerase chain reaction) amplified DNA from a chromosome that does not contain this sequence, and with probes containing dispersed repetitive sequences. The results were compared with Giemsa banding, DNA late replication and *Fok*I *in situ* digestion patterns. This allowed further differentiation between the chromatin types of this species. Centromeric and NOR-associated heterochromatin as well as euchromatin were shown to be free of *Fok*I sequence repeats. Among the interstitial late replicating Giemsa bands, subdivided into 'marker' and 'additional' bands, most of the marker bands located at mid-arm positions were composed mainly or exclusively of tandemly arranged *Fok*I repeats. Some of the marker bands and nearly all of the additional bands located in the vicinity of centromeres were free of *Fok*I sequence repeats, of *Fok*I recognition sites, and possibly also of dispersed repetitive sequences. They are probably composed of specific, not yet defined, repetitive sequences.

Key words: chromosome banding, FISH, flow sorting, *Fok*I sequence repeats, heterochromatin, *Vicia faba*

Introduction

Heterochromatin, as originally defined by Heitz (1929) for liverwort chromosomes, is positive heteropycnotic chromatin which remains more condensed than euchromatin during nuclear interphase. It is present in all tested eukaryotic genomes and detectable by different cytogenetic methods such as cold treatment, various Giemsa and fluorescence banding techniques or by differential incorporation of base analogues. Heterochromatin frequently proves to be genetically inert, late replicating, and composed of satellite or highly repetitive simple sequence DNA. Even within a species different types of heterochromatin such as constitutive, facultative or functional heterochromatin (Nagl 1976) may occur.

In the field bean, *Vicia faba*, Giemsa banding revealed a comprehensive pattern of heterochromatic bands, categorized by Döbel *et al.* (1978) into thin centromeric bands, the nucleolar organizer region (NOR)-associated heterochromatin, intercalary 'marker bands' and the smaller, less regularly occurring, 'additional bands'. The DNA of most of the intercalary marker and additional bands proved to be late replicating, while the NOR was the earliest replicating segment of the genome (Döbel *et al.* 1978, Schubert & Rieger 1991). Many of the marker band regions appeared as 'extended segments' after incorporation of the base analogue deoxyazacytidine (Fucik *et al.* 1970).

Kato *et al.* (1984) described a 59–bp repeated *Fok*I element of the field bean genome. The location of these repeats within the heterochromatic regions of the long arms of the five pairs of acrocentric chromosomes was determined by radioactive *in situ* hybridization (Yakura *et al.* 1987). *In situ* digestion of *V. faba* chromosomes with the restriction enzyme *Fok*I prior to Giemsa staining (Schubert 1991, Schubert *et al.* 1993) indicated that late replicating interstitial Giemsa bands may be subdivided into two groups. One is completely cleaved by *Fok*I and the other is more resistant than the remaining euchromatin, the centromeric heterochromatin and the NOR.

In the present paper, we show that the *Fok*I-sensitive heterochromatic bands are identical to the regions which exclusively hybridize to the biotinylated *Fok*I sequence repeat, while the other interstitial heterochromatic Giemsa banded regions are free of this sequence and apparently composed of other repeats which also deviate from dispersed repetitive sequences.

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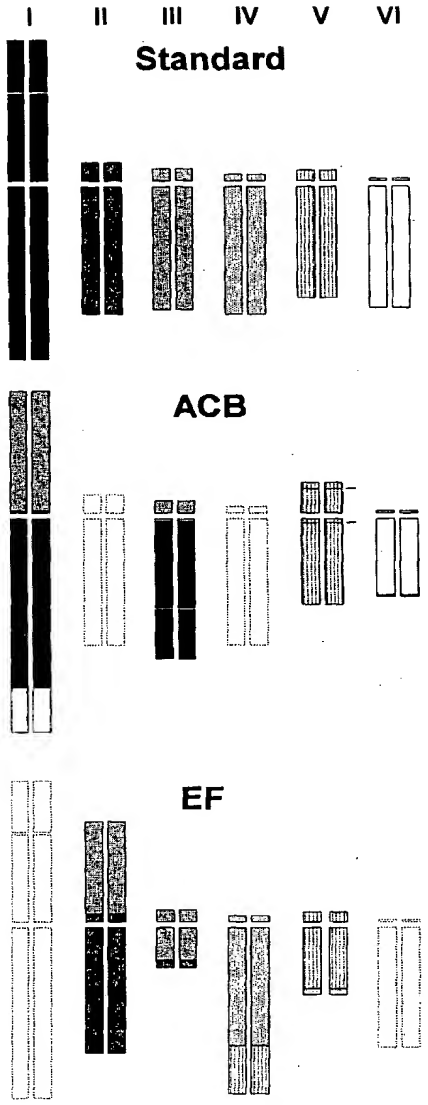

Figure 1. Scheme of the standard (wildtype) and of the reconstructed *Vicia faba* karyotypes ACB and EF. Changes of bitmap pattern mark the composition of translocated chromosomes of karyotypes ACB and EF. Breakpoints that led to the pericentric inversion of chromosome V of karyotype ACB are marked by bars.

Material and methods

Chromosome suspensions were obtained according to the methods of Schubert *et al.* (1993) from synchronized root tip meristems of two lines with reconstructed karyotypes and individually distinguishable chromosome pairs. (For description of the lines ACB and EF see Figure 1, and Döbel *et al.* (1978) and Schubert *et al.* (1982)). The chromosome suspensions were either dropped on slides for fluorescent *in situ* hybridization (FISH; Lichter & Cremer 1992) or used for flow sorting of chromosomes by a FACStar Plus (Becton & Dickinson) according to Lucretti *et al.* (1993).

The *Fok*I repeat was amplified from genomic DNA of *V. faba* by PCR with a sequence specific primer pair (5'-GCCTTGTCATTATGGAAGGTAGTCTG-3' and 5'-CCATCCATTGGAGTAACAAAAACTTCG-3') comprising positions 54–20 and 51–25 of the repeat (Kato *et al.* 1984). After *Fok*I cleavage the amplification products showed a ladder pattern of multimers of the basic repeat on agarose gel (Kato *et al.* 1984). These PCR products were reamplified for labelling with Bio-11-dUTP using the following protocol.

Primary *Fok*I repeat amplification was performed using 40 pg genomic DNA in a final volume of 50 µl, in 1 × *Taq* polymerase buffer (Promega), 1 mM $MgCl_2$, 0.2 µM primer, 0.2 mM of each dNTP. DNA was denatured at 94°C for 10 min and after cooling to 85°C, 2.5 U *Taq* DNA polymerase were added. Thirty cycles followed at 55°C for 30 s, 67°C for 30 s, and 94°C for 30 s. For labelling, 2 µl of the primary amplification volume were used in a two-step asymmetric PCR. The first 30 amplification cycles were performed in a final volume of 100 µl containing 1 × *Taq* polymerase buffer, 1 mM $MgCl_2$, 0.22 µM of one of the *Fok* primers, and 0.2 mM dNTPs, except dTTP. dTTP (150 µM) and 150 µM biotin-11-dUTP were added for labelling. After denaturation, 5 U *Taq* polymerase were added and 30 cycles at 55°C for 30 s, 67°C for 50 s and 94°C for 30 s followed. For the second amplification 0.1 µM of the missing second *Fok* sequence-specific primer and 5 U *Taq* polymerase were added. Ten cycles at 55°C for 50 s, 72°C for 50 s, 94°C for 50 s, and a final extension step at 72°C for 5 min followed.

DNA of flow sorted chromosome III of line EF was amplified by PCR using degenerated oligonucleotide primers (6-MW primer; Telenius *et al.* 1992a,b) as described (Pich *et al.* submitted) and labelled for FISH by PCR using 1 × *Taq* polymerase buffer, 2.5 mM $MgCl_2$, 0.7 µM 6-MW primer, 0.2 mM dNTPs, except dTTP. For labelling, 150 µM dTTP and 150 µM Biotin-11-dUTP were added. After denaturation, 25 cycles at 55°C for 1 min, 72°C for 1.5 min with an extension of 1 s per cycle, 94°C for 1 min and a final extension at 72°C for 10 min followed.

Genomic clones of an unknown seed protein (USP; Bäumlein *et al.* 1991) and legumin B4 (Heim *et al.* 1989) were biotinylated by nick translation (Langer *et al.* 1981) and used for FISH.

Results and discussion

In situ hybridization with biotinylated *Fok*I sequence repeats produced distinct chromosome-specific signal patterns after detection with streptavidin-fluorescein isothiocyanate (streptavidin-FITC) and

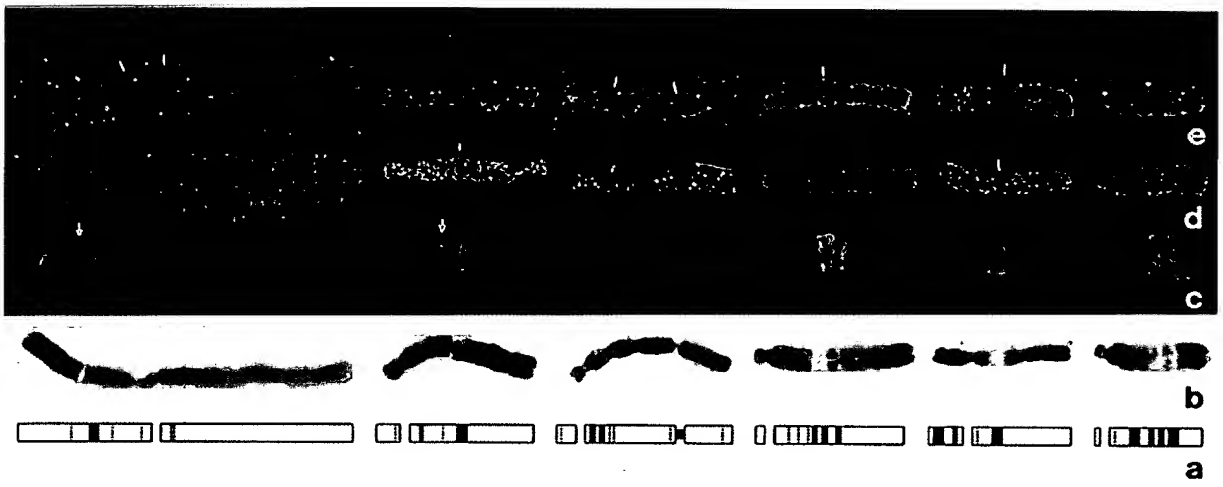

Figure 2. Correlation of Giemsa bands. *Fok*I digestion pattern and *in situ* hybridization signals in chromosomes of karyotype ACB. **a** Schematic chromosomes with Giemsa marker bands (black) including the NOR and additional interstitial bands (dashed). **b** Dark and light bands after *in situ* digestion with *Fok*I prior to Giemsa staining. **c** Signals after *in situ* hybridization with biotinylated *Fok*I sequence repeats (the small dot-like signals are indicated by arrows). **d** Signals after *in situ* hybridization with DOP-PCR products from DNA of flow sorted chromosome III of karyotype EF (Figure 1). Note the absence of signals over the area of the Giemsa marker band in the long arm of chromosome V. **e** Signals after *in situ* hybridization with a genomic clone (LeB4, Heim *et al.* 1989) containing the legumin B4 gene and ~12 kb flanking repetitive sequences. The absence of signals over the areas of the marker band in the long arms of chromosome V, and reduction of signals in regions of marker bands of chromosomes I, II, III, IV and additional bands close to centromere of chromosome I are marked by bars in d and e.

amplification with biotinylated antistreptavidin (Figure 2). The short arm of chromosome I, which represents most of the long arm of the wildtype chromosome III, showed two adjacent signal bands and a distal dot-like signal. These signals correspond to the heterochromatic marker band (in less condensed chromosomes detectable as two bands) and an adjacent additional band. Chromosome II showed two close signals in the middle of the long arm coincident positionally with the frequently fused marker band(s) and a weaker signal covering the proximal additional band. Chromosome III, composed of the short arm and centromere of wildtype chromosome III and the satellite arm of the wildtype metacentric chromosome I, showed no signal, in common with the *in situ* *Fok*I digestion data. Chromosome IV revealed three signal bands coincident positionally with the three marker bands, and sometimes a distal pair of signal dots. The pericentrically inverted chromosome V showed the most pronounced signal band covering the marker band of its long arm. Chromosome VI contained three signal bands representing the three distal marker bands.

With the exception of the marker bands in the satellite arm, in the short arm of chromosome V and the proximal marker band of chromosome VI, all late replicating marker bands were composed of *Fok*I sequence repeats. These results are in accordance with the complete *in situ* digestion of these regions by *Fok*I. Only the dot-like signals located distal to the marker band(s) of the short arm of chromosome I (the long arm of wildtype chromosome III) and proximal to the marker band(s) of chromosome II, which coincide in position with additional bands, were previously not resolved as negative bands after *Fok*I digestion, probably due to their small dimension. The same is true for the infrequent signal dots distal to the marker bands of chromosome IV.

All unlabelled additional and marker bands, except the band at the NOR, remained darkly Giemsa-stained after restriction enzyme treatment (Figure 2), i.e. they were resistant to *Fok*I cleavage. The moderate digestion of the remnant of the genome is obviously due to dispersed *Fok*I recognition sites which occur independent of *Fok* sequence repeats.

The absence of clustered *Fok* repeats from other than the labelled regions is indicated by a further experiment. Chromosome III of the reconstructed karyotype EF is composed of the short arm, the centromere and the proximal part of the long arm of wildtype chromosome III and the distal half of the short arm of chromosome II and does not include any region highly sensitive to *Fok*I cleavage. When DNA of this chromosome was amplified by PCR with *Fok*I-specific primers no *Fok* repeats were detectable. However, after DOP-PCR with DNA from this chro-

mosome as a target, products were obtained that after biotinylation and *in situ* hybridization, label the whole chromosome complement, except for the regions containing *Fok* repeat clusters. The lack of signals is especially impressive for the marker band region of the long arm of chromosome V (Figure 2). Comparable results were obtained by *in situ* hybridization with dispersed repetitive DNA flanking genes which code for the seed proteins USP and legumin B4. In these experiments not only regions containing *Fok* sequence clusters but sometimes also the marker bands of the satellite arm which seemed to be free of *Fok* sequence repeats revealed reduced signal densities (Figure 2).

Conclusions

The DNA of centromeric and NOR-associated heterochromatin and of the euchromatin of *V. faba* is cleavable by *Fok*I and does not contain detectable amounts of *Fok* sequence repeats. The DNA of some marker bands and nearly all additional bands, especially those in the vicinity of the centromeres, is free of *Fok* sequence repeats and not cleavable by *Fok*I. At least some of the marker bands (and additional bands) seemed to be free also of dispersed repetitive sequences and are probably composed of (a) specific not yet defined repetitive sequence(s). Most of the late replicating Giemsa marker bands in mid arm positions seem to be exclusively composed of tandemly arranged *Fok* sequence repeats.

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References

Bäumlein H, Boerjan W, Nagy I, *et al.* (1991) A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants. *Mol Gen Genet* **225**: 459–467.

Döbel P, Schubert I, Rieger R (1978) Distribution of heterochromatin in a reconstructed karyotype of *Vicia faba* as identified by banding- and DNA-late replication patterns. *Chromosoma* **69**: 193–209.

Fucik V, Michaelis A, Rieger R (1970) On the induction of segment extension and chromatid structural changes in *Vicia faba* chromosomes after treatment with 5-azacytidine and 5-azadeoxycytidine. *Mutat Res* 9: 599–606.

Heim U, Schubert R, Bäumlein H, Wobus U (1989) The legumin gene family: structure and evolutionary implications of *Vicia faba* B-type genes and pseudogenes. *Plant Mol Biol* **13**: 653–663.

Heitz E (1929) Heterochromatin, Chromocentren, Chromomeren. *Ber Dtsch Bot Ges* **47**: 274–284.

Kato A, Yakura K, Tanifuji S (1984) Sequence analysis of *Vicia faba* repeated DNA, the *Fok*I repeat element. *Nucleic Acids Res* **12**: 6415–6426.

Langer PR, Waldrop AA, Ward, DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc Natl Acad Sci USA* **78**: 6633–6637.

Lichter P, Cremer T (1992) Chromosome analysis by non-isotopic *in situ* hybridization. In: Rooney DE, Czepulkowski BH, eds. *Human Cytogenetics: A Practical Approach Vol I*, 2nd edn. Oxford, IRL Press, pp 157–192.

Lucretti S, Dolezel J, Schubert I, Fuchs J (1993) Flow karyotyping and sorting of *Vicia faba* chromosomes. *Theor Appl Genet* **85**: 665–672.

Nagl W (1976) *Zellkern und Zellzyklen – Molekularbiologie, Organisation und Entwicklungsphysiologie der Desoxyribonukleinsäure und des Chromatins*. Ulmer, Stuttgart, p 486.

Pich U, Houben A, Fuchs J, Meister A, Schubert I (1993) Utility of DOP-PCR amplified DNA from total genome and defined chromosome regions of the field bean. *Chromosome Res*, submitted.

Schubert I (1991) *Fok*I digestion of broad bean chromosomes causes a specific banding pattern. *Biol Zentbl* **110**: 195–198.

Schubert I, Rieger R (1991) Characterization of plant chromosomes by means of banding techniques, differential incorporation of base analogues, and *in situ* hybridization. In: Obe G, Sobti A, eds. *The Eukaryotic Chromosome – Structural and Functional Aspects*. New Dehli, Springer-Verlag, pp 31–46.

Schubert I, Michaelis A, Rieger R (1982) Karyotype variability and evolution in *Vicia faba* L. *Biol Zentbl* **101**: 793–806.

Schubert I, Dolezel J, Houben A, Scherthan H, Wanner G (1993) Refined examination of plant metaphase chromosome structure at different levels made feasible by new isolation methods. *Chromosoma* **102**: 96–101.

Telenius H, Carter NP, Bebb CE, *et al.* (1992a) Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* **13**: 718–725.

Telenius H, Pelmear AH, Tunnacliffe A, *et al.* (1992b) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes, Chromosomes, Cancer* **4**: 257–263.

Yakura K, Kato A, Tanifuji S (1987) Cytological localization of highly repeated DNA sequences, the *Fok*I sequence family and *Bam*HI sequence families, in *Vicia faba* chromosomes. *Jap J Genet* **62**: 325–332.

Note added in proof

In subsequent experiments we investigated the closely related species *Vicia narbonensis* with respect to *in situ* digestion with *Fok*I endonuclease, and also *in situ* hybridization with labelled *Fok*I elements. Digestion was incomplete and no significant hybridization was observed in either eu- or hetero-chromatin. Thus, *Fok*I elements seem to have evolved only after phylogenetic separation of the field bean.